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It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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SOME BACTERIOLOGICAL ASPECTS OF DEHYDRATION[1]

SAMUEL C. PRESCOTT

Massachusetts Institute of Technology

One of the most striking phenomena of the world war period—through which we have, we hope, now happily passed—is the great stimulation which has taken place in all branches of pure and applied science. While during this half-decade discovery and invention have been especially directed in many scientific fields to the development of more terrible methods of destruction of human life and property, the energies of those in our own profession have been largely turned toward the constructive side, in the alleviation of pain and disease, the control of infections, and the development of means to promote rather than retard the welfare of the individual and the race. We have witnessed the tremendous expansion which has taken place in chemistry, in aeronautics, in war engineering of all kinds, and we have also seen and participated in the vast but perhaps less spectacular developments in our own field of bacteriology—developments which will attain their complete flower and fruition as arts of peace rather than of war. It is only necessary to review the work of the past two or three years, as evidenced by our own former programs, to observe the tremendous impetus that the war has given to medical and public health bacteriology. The somewhat more restricted field of industrial bacteriology and its applications, as in fermentation, soil bacteriology and the preservation and conservation of foods, while less conspicuous, has, however, shared in the general growth of the science that

[1] Address of the President of the Society of American Bacteriologists delivered at twenty-first annual meeting, Boston, December 29, 1919.

has taken place, and gives promise of an even greater development as a result of the demands of commerce and industry. Processes which five years ago were thought of merely as interesting possibilities, such as the production of glycerin and acetone by fermentation, have now been so perfected that they may compete with purely chemical methods. The manufacture of numerous organic bodies is now possible, and agriculture seems likely to receive its next great impetus as a result of advances which will be made in the bacteriological investigations of soils. Food conservation has never occupied so important a position, from the standpoint of world welfare, and here, too, advances of great import have been made. It is of one phase of this subject that I wish especially to speak at this time.

In days of peace, with new countries opening up and rapidly extending their agriculture, with larger crops, better knowledge of utilization of fertilizers, improved methods of food handling and better transportation facilities, we have been inclined to regard the dangers of general food shortage or starvation except in restricted localities as imaginary and impossible, and to believe that our system of crop production and marketing, storage and conservation, while not fully utilizing the scientific knowledge available, would at least sufficiently safeguard the world from hunger or serious economic disturbances. War conditions have modified our points of view very greatly, however, and we have been awakened to the importance of the principles expressed by Malthus in 1798, or at least a modification of his thesis, pertaining to the relation of food supply to population. Now we are more appreciative than ever before of the dangers which may and actually do threaten us because of waste, unscientific methods of production and conservation, and improperly coordinated regulation of supply and demand. The war years through which we have just passed have caused us to give serious attention to the problems of food supply and food control. It has fallen to this country to be the storehouse from which enormous supplies of foods have been withdrawn for the use of the fighting forces and civilian populations of Europe. While this demand has been largely for meats and cereals, and especially for wheat, it has

also affected almost every type of food substance. Because of the necessity for sending these enormous quantities of breadstuffs abroad it has been especially desirable that the problems of food conservation in this country should be studied with especial care, to utilize as fully as possible all those methods of food treatment which would make it possible to lessen waste and build up a reserve against another period of shortage, bad crops, or unusual exportation. These investigations had their first fruits in the movement for war gardens and home canning and preserving, and their later and more permanent results in the stimulation of renewed interest in the great industrial methods of food preservation, and particularly in the impetus given to drying as a means of protecting foods against spoilage, and preserving them for future use. Since the problem of spoilage is essentially a microbiological problem, it follows that the investigation of dehydration has manifold bacteriological aspects.

Dehydration, desiccation, or drying as applied to foods, may be defined as the process of removal of the surplus water from the food substance in such a way as to prevent destruction of the cellular tissues, maintain the energy values, and preserve potentially the color, flavor and physical condition of the food. Since practically all foods, with exception of the ripe seeds of cereals, legumes, and a few other plants, contain a large percentage of water and as a result have relatively short storage periods in the fresh state, it is clear that the rapid, regulated drying of such foods will, if it can be successfully accomplished, add greatly to the world's stock in a form which is capable of easy storage, favorable transportation and universal employment. Moreover, when we appreciate that approximately 50 per cent of the total food produced in America in a single year never reaches the consumer because of poor crop management, bad market conditions, distance from means of transportation or losses en route and in the city markets, the great possibilities of dehydration begin to be apparent. Much could be said on the economic side of this subject, but at this time I desire to emphasize especially those phases which are of direct interest to us as bacteriologists, physicians or sanitarians.

Dehydration however is not a new art, for drying has been known for hundreds of years, and is probably the oldest method of food preservation which the human race has employed. It was undoubtedly known to the ancients, in fact it must have been practised as soon as man became a food-producing and food-storing animal. In arid regions this could be accomplished with little difficulty. The Indians of the plains and later the early settlers, dried their beef or buffalo meat by cutting it into thin strips and hanging them up for the sun and wind to remove the excess moisture and sear the surface with a protective coating which would prevent infection and spoilage. The material so dried was known as jerked beef. Our early Massachusetts colonists dried corn after it had been cooked, the product being known as samp. Native fruits and berries were dried in considerable quantity, especially the apple. Along the coast fish drying became an important industry, and throughout New England to the present time will be found the application of this process of food preservation as a sort of local or primitive industry, with little exact knowledge of the underlying principles. Similarly, in other parts of the country other fruits, vegetables and meat products have been dried. Peas and sweet corn may be mentioned as examples of the former, while along the Pacific coast the long sunny dry period lends itself particularly to the drying of prunes, raisins and other sweet fruits. That the results have now always been highly satisfactory cannot be gainsaid, for local differences in method, and differences in season have not infrequently been the cause of losses, spoilage and inferior products.

Dried or evaporated fruits have been in use in America for many years and have become an important part of our food products. On the other hand, dried vegetables have, until recently, been prepared only on a small scale, although the drying of potatoes was done on a semi-commercial scale in 1886 by A. F. Spawn, an American, resident at that time in Australia. The history of commercial and semi-commercial drying of vegetables in America is an interesting one, but the industry has not until the war period been a success financially, probably owing to the abundance of fresh materials, and to the prejudices which are

prone to develop in the public mind when new food products are put upon the market. The war, however, has changed the attitude of the public in these matters, and it is probable that there will soon be a considerable expansion of the dehydration industry in this country if the products can fulfill the commercial, nutritional and sanitary requirements.

Although somewhat aside from the main line which I wish to discuss it may be of interest from the standpoint of general information to observe how dehydration has been utilized in other countries. Little has been done in England or France. In Switzerland there are a few small concerns engaged in the drying of vegetables. In Germany dehydration of foods has had a great expansion since 1900 and to this process may be ascribed at least a portion of the success that country had in meeting the food situation during the war. In 1898 there were three small plants with an insignificant output, largely potatoes and other root vegetables. In 1906 the number of plants in operation had increased to 39, in 1909 to 199, and in 1914, at the outbreak of the war, to 488; in 1916 there were 841 drying plants and in addition about 2000 breweries were using some portion of their equipment in the drying of food materials. By 1917 the number of drying plants had again more than doubled, and the quantity of potatoes alone dehydrated in Germany was more than three times the total crop of the United States. This fact alone suggests one of the reasons why Germany was able to maintain her food supplies during the war. It also by inference supports the claims that have been made that drying does not seriously impair the nutritive qualities of foods, a point to which I may later return, although the bearing is not essentially bacteriological.

Let us now turn to some of the biological questions involved in this process of food preservation, considering first the relation of the water content to the growth of microörganisms. Although it has long been recognized that most microörganisms thrive but poorly in substances of low water content, even if those substances are rich in nutrients, definite data on this point are surprisingly difficult to secure. The general impression of the bacteriologist who is familiar with the general literature is that there is much

information on this subject. Search reveals relatively few papers, however, in which the studies of the growth of bacteria in food substances of high concentration have been reported, and even fewer dealing with the development of yeasts and molds. A few of these may be mentioned.

Eschenhagen found that as the concentration of the substratum increased fungi growing thereon modified their vegetative character, and especially developed swollen tips in the young hyphae.

Rasciboski observed wall thickenings, giant cells, and multiplication of nuclei.

Puriewitsch, in investigations on the growth of *Aspergillus pseudo-clavatus* in concentrated sugar solutions found that the walls became swollen, and the filaments developed the appearance of a string of pearl beads.

Klebs, in investigations on *Aspergillus repens*, found the limits of concentration which the organisms could withstand were 95 per cent for grape sugar and 57 per cent for glycerin. Similar results have been obtained by other investigators using fungi as the organisms studied. Perhaps the most interesting and illuminating of the investigations on this subject have been done by our fellow members, the Kopeloffs, in their studies of deterioration of cane sugar. They have found that in sugars containing as little as 1 per cent of water slight decomposition by fungi could take place. Other investigations which need not be enumerated in detail here have shown that many fungi have the ability to develop in or upon substances almost devoid of water, and our common experience with food substances and fabrics bears witness on this point.

With yeasts and bacteria, high concentration is apparently much more inhibitive than with the thread fungi, although these organisms too, can remain alive and sometimes increase slowly in and upon substances containing small amounts of water. Obviously, the character of the substance, its reaction and chemical composition have much to do with the ability of the organisms to remain viable.

Laurent found that certain yeasts would grow in and ferment 60 per cent sucrose solution. I have personally observed similar phenomena.

Working with bacteria, Zopf found that *Bact. vernicosum* would produce fermentation of 70 per cent sucrose in a mixture with cottonseed meal, and that *Staphylococcus aureus* destroyed cane sugar gelatin in a concentration of 48 per cent.

Grafenhan showed that certain bacteria will grow on 70 per cent cane sugar and dextrine.

The Kopeloffs, again, have confirmed or even exceeded these figures.

It is interesting to note on this point that cane sugar, or sugars, have generally been the subject which have been used for tests.

Although bacteria generally tend to disappear or at least to become inactive when the moisture falls below 40 per cent, there are many instances known when percentages of water much smaller than this will still permit growth. The ordinary evaporated fruits, such as apples, peaches and apricots contain in general about 20 to 24 per cent moisture. While usually this high concentration is sufficient to inhibit development, it sometimes requires but a small addition of moisture to permit growth and consequent deterioration by molds and yeasts or their enzymes. It must especially be borne in mind that the enzymes of these microörganisms, as Kopeloff has quantitatively proved, may be active even in the presence of minute moisture percentages, and that they may bring about marked deteriorations. This we have also found to be true in certain dehydrated products.

Resistance to drying and ability to increase in the presence of small percentages of water *seems* to be particularly characteristic of soil bacteria and those native to vegetables themselves while most microörganisms of pathogenic character, accustomed to the fluids of the body have fortunately much less endurance to dryness. The importance of this fact (if it proves to be a fact) in the preparation of dehydrated food substances cannot be overestimated.

BACTERIOLOGY OF DEHYDRATED VEGETABLES

It was my good fortune, during the period of the war, when I was in the Food Division of the Surgeon General's Office, to have assigned to me a certain part of the investigation work on new

food materials and the various food compounds and substances which were brought to the Surgeon General's Office for examination. Among these were the dehydrated vegetables which were later purchased in considerable quantity and used by our forces on the other side. Although the quantity actually used was small as compared with the total food supply, you may be interested to known that something like forty thousand tons were sent over. In connection with investigations on this subject, I thought it desirable to make certain bacteriological studies of these foods, which I wish to report upon at this time.

Dehydrated vegetables generally contain less than 10 per cent of water. They have often been subjected to a pre-treatment by hot water or steam and to more or less thorough washing. The drying temperatures vary from 130° to 185°F. (55° to 85°C.) with different processes, and the heating period is of 3 to 24 hours' duration, although rapid evaporation may considerably reduce this during a portion of the time.

It may be further noted that these dehydrated substances will, when soaked in water, reabsorb the water which has been lost and assume the normal condition of the tissues. On the central table there are some specimens of dehydrated products which have been restored by simply adding water, and at the end of this meeting I should be very glad to have you examine the dried and restored substances as they appear there.

The presence of bacteria in or upon dehydrated vegetables may therefore be of some interest. They may be:

1. Organisms originally on food which have survived the process (and perhaps increased).

2. Organisms gaining access from wash water, handling in raw state, or utensils.

3. Organisms deposited on surfaces during packing, handling in dried state, or utensils.

Studies on a large number of samples of dried foods have shown very few, if any, of them to be sterile, although often the numbers of bacteria present were very small. Moreover, long continued keeping under suitable conditions causes the number to decrease gradually. Mold spores were commonly present, but yeasts

have invariably been absent. The methods of quantitative determinations are at best imperfect because of the character of the materials examined. It was found necessary therefore to make multiple examinations, and derive the "normal" figure from the results obtained. The nature of the materials studied rendered it impossible to extract, plate and count all the organisms present, but it was possible to secure a result which showed the general order of magnitude of the microbic population. The method found most satisfactory was as follows:

Ten grams of the sample, well broken up, with aseptic precautions, were weighed out into a tared sterile dish and later transferred to an Erlenmeyer flask containing 200 cc. of sterile tap water. After thorough mixing in order to get all the particles completely wetted, the sample was placed in a 37° incubator for two hours to permit absorption of water, return of tissues to as near normal as possible and easy separation of bacteria. At the end of this time the sample was again thoroughly agitated and aliquot portions removed, dilutions prepared and plates made as rapidly as possible. The two hour period of infusion was determined upon as a result of preliminary studies. Soaking is necessary to make it possible to remove the bacteria from the surfaces of the particles, but too long continued infusion permits the growth of bacteria which have been restored from the plasmolysed condition.

The media which were found most satisfactory for quantitative studies were plain agar and glucose agar or litmus glucose agar for the bacteria, and Czapek's medium for fungi. Plating on beer wort gelatine or agar was carried out for a time for the purpose of enumerating yeasts, but the results were so uniformly negative that this process was discontinued.

Incubation at 37° for forty to forty-eight hours with plates inverted was found to be most satisfactory. Counts were made with a reading glass, the dish being placed on a black ruled plate. Examination of each plate was made for predominant types of colonies, and these were fished and later identified in order to determine as fully as possible the source and kinds of organisms.

Products from a number of different manufacturers were examined, and these varied greatly in results, in part because of the different methods of treatment and in part, no doubt, because of differences in sanitary conditions of plant and methods of handling. The chart here shown may serve to show the general character of the numerical results obtained.

NUMBER	MATERIAL	MOISTURE	BACTERIA PER GRAM			MOLDS PER GRAM
			Total P. agar	Gluc. agar	Acid	
		per cent				
1	Potato	3.14	9,400	9,800	3,800	60
2	Carrot	4.42	23,000	24,000	8,600	20
3	Cabbage	5.93	32,000	340,000	240,000	600
4	Turnip	5.62	220,000	300,000	20,000	20
5	Mixed vegetables	5.60	1,600,000	2,000,000	2,000,000	80
11	Turnip	5.00	3,800	7,000	3,000	
12	Cabbage	6.24	7,000	8,000	2,800	200
13	Onion	5.73	2,200	2,400	1,000	> 20
14	Potato	2.78	6,800	6,900	1,500	> 20
16	Tomato	3.32	26,000	5,000	1,000	> 20
21	Beet	3.65	700	800		60
22	Banana	4.72	600	600		> 20
23	Sweet potato	4.42	100	900		100
24	String beans	4.27	600	800		> 20
27	Peaches	2.79	800	200		40
31	Carrots	5.85	80	200		> 20
32	Onions	4.03	400	6,000	2,000	400
33	Parsnips	4.09	4,400	18,000	10,000	60
34	Turnip	7.08	60	180		> 20
36	Potato	2.69	400	1,200		440

> means less than.

The foregoing table serves to show the variations found in the total numbers of organisms occurring on dehydrated products. Study of the types predominating has given no evidence of objectionable organisms, practically all the species found having their habitat in soil or water. The following species were identified: *B. vaculatus, B. denitrificans, B. Lustigi, B. cuticularis, B. weichselbanini, Ps. cohaerea, Ps. sinuosa, B. subflavus, B. tenacatis, B. ginglymus, Bact. mycoides, M. radiatus, Ps. viridescens, Ps. ambigua.*

The molds isolated were identified as of the following genera: Aspergillus, Mucor, Penicillium, Spicaria, Sporotrichum, Trichoderma, Herpocladiella.

At least two species of Mucor, two of Penicillium and five of Aspergillus were isolated. These are all organisms which may be found on fresh vegetables or fruits. Probably the spores survive all pretreatment and dehydrating processes.

From the standpoint of numbers and types of bacteria, these dehydrated products have the same characteristics as raw fruits and vegetables, except that the number of organisms has been greatly reduced by the processes to which the foods have been subjected.

STORAGE OF DEHYDRATED PRODUCTS AND EFFECT OF STORAGE ON BACTERIAL CONTENT

The practicability of dehydrated foods must depend largely on whether they may be held in storage for long periods without undergoing any deteriorations. If, as has been claimed, they may be kept under all kinds of conditions if properly sealed, their great usefulness is unquestioned, but the type of container becomes of supreme importance. To determine this point an extended study has been made during the past two years to determine the effects of various conditions of storage on the quality of the product.

As the preservation of dehydrated vegetables depends upon the inhibitive effect of their low moisture content upon any destructive bacteria or other organisms which may be present, it is obvious that the greatest difficulty of preservation would occur where the products were exposed to a high relative humidity, permitting them to absorb moisture and at the same time exposed to a temperature which was most favorable to the growth of organisms after the inhibition of a low moisture content was removed. Consequently, in order to subject samples of the dehydrated foods to the severest conditions they would be likely to encounter in transporation, storage, or as merchandise, a series of investigations was carried out with the purpose of determining the effect

of the environment on the bacteriological character of the foods themselves. Four types of storage conditions were employed. The first is called "*severe*," since the chamber used was kept at 37°C. and the relative humidity at 100 per cent. The second was "*moderate*" in character, the temperature being 20° to 25°C. with a relative humidity of about 70 per cent. Third, "*favorable*" storage, with chamber maintained in one case at ordinary temperature and in another at 37°C. but with no artificial humidification. In both, the relative humidity was approximately 50 per cent. This condition represents fairly well the conditions which may be met in ordinary transportation and commercial handling. The fourth condition employed was "*cold storage*" at 0°C. and about 95 per cent relative humidity.

Six weeks was taken as the maximum period of storage under the various conditions with the first set of products. Examination of the products was made at the end of each two weeks.

Since this study is a combination of the sanitary and the commerical or industrial phases of the subject, we have deemed it desirable to use as containers for the material under investigation a variety of the newer types of treated paper cans or cylinders as well as tinplate and glass. It is obvious that in tightly stoppered glass containers and in tin cans sealed or tightly closed by friction tops, there is no noteworthy opportunity for additional moisture to gain entrance. Any changes in the food product must therefore be the result of bacterial or enzymic action taking place at low moisture percentage. With the paper containers, on the other hand, the exclusion of moisture is far from perfect, even in those holders treated by paraffin, waxes, etc., to secure impermeability. Statements are often made that ordinary paper bags will serve to preserve dried vegetables and fruits, and while this is often true if storage conditions are dry and temperature low, it is on the other hand, often fallacious, especially in damp climates and with vegetables containing sugars or other hygroscopic ingredients.

Without going minutely into the methods of experimentation which have been followed, I wish to place before you the general results obtained.

Vegetables of the same kind and lot, thoroughly mixed, were subjected in the different types of containers to the four different sets of conditions which I have described. Initial determinations of moisture and microbic content were made, and the samples properly sealed, were left undisturbed for two, four and six weeks. At the end of this period new sets of determinations were made and the material subjected to careful examination for evidence of discoloration, loss of flavor and enzymic activity. Any deviation from normal appearance was recorded. While very small increases in moisture may not readily be detected microscopically, any really appreciable increase is evidenced by the changed appearance and texture of the material. Bacterial changes cannot be easily detected unless the increase is enormous, but comparatively slight development of mold is at once apparent.

Our data may be summarized as follows: Materials stored in tin and glass containers under all conditions of temperature and moisture remained practically constant in their moisture content and mold content. The bacterial counts generally diminished, sometimes rapidly, sometimes gradually. In other words, bacteria with proper storage conditions die off slowly.

Samples stored at 37°C. and high humidity in paper containers, even though they were heavily treated with paraffin showed little or no increase in moisture at the end of two weeks, and often had not doubled their percentage of water in four weeks, whereas in six weeks, the amount of moisture often increased by 400 per cent or even in some instances by 1000 per cent. It is a strange fact, but apparently a fact, that atmospheric moisture will penetrate very rapidly through the paraffin containers if exposed four to six weeks, while some containers could be immersed in water and opened at the end of that time without finding any visible water inside the container. The rate of absorption of atmospheric moisture by dehydrated vegetables when directly exposed to moist air is very moderate at first. The acceleration of absorption may be due to heightened permeability of the cell membranes as a result of slight swelling and increased conductivity of the fibrovascular bundles and other types of cellular structures during the early stages of absorption of water.

This increase in moisture content was quickly followed by changes in the flora of the samples. At first the bacterial count dropped somewhat and the fungus (mold) count remained practically constant, although the incipient development of mold mycelium was frequently apparent. At the end of four weeks the bacterial count was generally still low, although showing an upward trend in many instances, and the mold count (spores) had not greatly increased. Mycelium was, however, more marked. At the end of six weeks the bacterial count and the mold count had both increased greatly, although sometimes an interesting antagonism was exhibited, the bacteria falling off rapidly in numbers as the molds developed to large numbers and produced their characteristic cleavage and growth products.

It is apparent from the data obtained that ordinary paper containers do not protect dehydrated foods sufficiently at high temperatures and high humidity. Interestingly enough, similar results were obtained in cold storage (0°, 95 per cent humidity). The moisture content of the vegetables increased in six weeks by 400 per cent, the bacterial counts trebled while the molds remained nearly constant.

The samples stored in paper containers at ordinary temperatures and humidity showed little if any increase in moisture during the first four weeks, but at the end of six weeks it had somewhat increased. This was not sufficient to revive the dried up bacterial cells, or cause mold spores to germinate, and there was therefore no important increase in either of these groups of organisms. In this connection it may be stated that for certain molds and some bacteria, limiting moisture minima were observed, it being found that marked development did not ensue until the moisture of the sample had reached a definite percentage. These limiting minima vary from 22 to 32 per cent with different organisms.

Storage at 37°C. in a "dry" atmosphere differs slightly from that at 20°C. In general, there was a consistent decrease in moisture unless the material stored had been very thoroughly "dehydrated." By the end of six weeks the moisture had generally fallen to, or below, half the percentage of water in the original

sample. As would be expected, there was a decrease in the number of bacteria. The mold spores remained practically constant.

The practical result of this somewhat extended investigation was to prove beyond doubt that paper or wood pulp containers of good quality may be used with safety under the ordinary conditions pertaining in the temperate zones. Their cheapness and availability will help to establish the industry commercially. No invasion of organisms from outside takes place, and the variations in temperature and humidity of the outside are accompanied by a corresponding but perhaps smaller rise and fall in moisture in the food materials. Eventually an equilibrium with the air is reached. It is doubtful if the moisture would ever reach the point sufficient to bring about bacterial decomposition. On the other hand, these containers do not appear to be suitable for use in hot moist conditions for the reasons that have been pointed out through the results I have given.

DANGER OF FOOD POISONING OUTBREAKS THROUGH USE OF DEHYDRATED FOODS

Our attention has been directed during recent years to a number of cases of so-called food poisoning. These are now generally recognized as infections with some one or more of a number of organisms, among which may be named *B. paratyphosus A* and *B*, *B. enteritidis*, *B. welchii*, *B. suipestifer* and *B. botulinus*. The fact that dehydrated foods are not cooked or sterilized as they are prepared and packed for sale, brings up a question as to possible dangers from this source. Moreover, the methods of gardening employed in the production of vegetables for dehydration in some parts of the country, as in California, where Japanese gardeners produce them in large quantities, make it conceivable that the vegetables may reach the dehydration establishment infected with some of these bacteria or with *B. coli* or *B. typhosus*.

This matter seemed to be of sufficient practical importance to warrant a careful study of the effect of drying on these organisms. Two sets of experiments were conducted in the laboratory and one small investigation has been carried out in a dehydrating

plant under actual commercial conditions. In the laboratory tests stringless beans, parsnips, tomatoes and spinach were used. These vegetables were inoculated with cultures of the following organisms: *B. coli*, *B. typhosus*, *B. paratyphosus A* and *B*, *B. enteritidis*, *B. paracoli*, *B. subtilis* and *B. welchii*. The vegetables were dried in air in an oven at 80°C. for four hours, after which examinations were made to discover surviving organisms.

Of the organisms employed, *B. subtilis* was recovered in typical culture. Organisms resembling *B. typhosus* and the paratyphoid forms in some respects, but not typical, were found, but these have not yet been tested by serum reactions. All others were destroyed.

A second series has also recently been studied imperfectly in which *B. coli*, *B. paracoli*, *B. enteritidis*, the paratyphosus A and B strains, *Danysz bacillus*, *B. suipestifer* and *Microspira protea* and *B. diphtheriae* were used. The treatment given was the same as in the first test, four hours at 80° in an air oven. The only organisms recovered were atypical *B. typhosus* and atypical *B. suipestifer* and these have yet to be proved by serum reactions.

These experiments can only be regarded as preliminary, but so far as they go they are reassuring. It should here be stated that many vegetables contain on their surfaces organisms markedly simulating members of the colon typhoid group in gross appearance and in many reactions, as well as microscopically. Serum tests and animal tests may therefore prove the surviving types to be entirely different from the organisms used.

To make a more satisfactory investigation on this point, I have recently conducted in Chicago a set of experiments under actual working conditions in a dehydrating plant using a method which we had found to be highly satisfactory. The vegetables employed were carrots, cabbage and potato. The organisms used were: *B. typhosus*, *B. paratyphosus A*, *B. paratyphosus B*, *B. suipestifer*, *B. of Danysz* (*B. murisepticum*), *B. enteritidis*, *B. coli*, *B. paracoli*, *B. welchii*, *B. botulinus*, *Microspira protea* and *Micrococcus pyogenes aureus*. Heavy inoculations were made from rich broth or agar shake cultures, and the vegetables then submitted to the regular processes of pretreatment and dehydration.

The results of this work were most reassuring, since all the organisms, even *B. subtilis*, were destroyed, or at least so greatly reduced that they could not be discovered by subsequent culture examinations. These results must be regarded as preliminary and an extended study on the subject is projected for the near future.

There are many nutritional and commercial phases of dehydration, such as the effect on the so-called vitamines, which are of great interest, but would be out of place in a paper of this kind. I hope, however, that I have been fortunate enough to bring clearly before you the importance of this new-old method of food preservation, and to convince you that the products are not only excellent in appearance and appetizing in flavor, but are also safe and wholesome and free from the dangers of infections which may give rise to serious illness or death. The great advantages of fresh foods at all times and in all parts of the world, combined with the sanitary quality and safety which may be secured, should go far to promote this industry, while the economic advantages alone should make it worth while to foster its development along scientific lines. There are many studies yet to be conducted on the bacteriological side, and I trust that at some future meeting of this Society I may have an opportunity to present the results of later researches.

REPORT OF THE COMMITTEE ON THE DESCRIPTIVE CHART FOR 1919

H. J. CONN, *Chairman*, H. A. HARDING, I. J. KLIGLER, W. D. FROST, M. J. PRUCHA, AND H. N. ATKINS

Presented at the Boston Meeting of the Society of American Bacteriologists, December 29, 1919

PART I. METHODS OF PURE CULTURE STUDY. REVISED

The 1917 report of this committee contained a description of the methods recommended to be used with the Descriptive Chart. Copies of the report have been on sale by the Society and the first edition is now practically exhausted. Opportunity is therefore taken to revise the report, printing it in the JOURNAL OF BACTERIOLOGY to call it to the attention of the members of the Society, the reprints to be placed on sale as before. As soon as these reprints are available, one copy will be furnished with every order for Descriptive Charts, and additional copies may be obtained for 15 cents apiece postpaid, or if five or more copies are ordered, for 10 cents each, transportation not postpaid. Orders for these reports, as well as for the Descriptive Charts, are to be sent to the chairman of the committee (address Geneva, N. Y.).

Methods are given in this report for all the determinations listed on the 1917 chart (recommended for instruction) but methods have not yet been prepared for all the determinations called for by the older chart. As the chart designed for instruction is now in most general use, it has seemed wisest to give further attention to these few methods rather than to do work on some of the other, less frequently used, determinations. The methods given here are not to be considered official. They are merely the best that have come to the attention of the committee at the present time. Criticisms and suggestions are at all times welcome, in order that future editions of this report may be brought up-to-date.

PREPARATION OF MEDIA

Beef-extract broth shall have the following composition:

Beef-extract	3 grams
Peptone	5 grams
Distilled water	1000 cc.

Beef-extract agar shall be of the same composition plus the addition of 12 grams of oven-dried agar or 15 grams of commercial agar. *Beef-extract gelatin* shall be of the same composition, with the addition of 120 grams of Gold Label gelatin, or 100 grams of some brand of gelatin (such as "Bacto-gelatin," or United States Glue Company gelatin) having greater jellying power. These media are to be made up according to the directions given by the committees of the American Public Health Association on water analysis and on milk analysis (1916, 1917), except that white of egg may be used for clarification if desired. It is recommended, however, that instead of using phenolphthalein in adjusting the reaction of these media the simpler and more accurate procedure be adopted of adjusting to the neutral point of brom thymol blue.[1] Bring the media to such an acidity as to turn this indicator a distinct grass-green (neither yellow green nor blue green). This color indicates approximately "true neutrality," i.e., a hydrogen-ion concentration between pH = 6.6 and pH = 7.4. Another equally satisfactory method of adjusting media to this hydrogen-ion concentration is to bring them to such an acidity as to cause the first faint trace of permanent pink to appear with phenol red.[2]

Sugar broths. Just before sterilization 1 per cent of the required carbohydrate is to be added to beef-extract broth. Otherwise proceed as for sugar-free broth. Adjust reaction with brom thymol blue or phenol red.

Plain gelatin. Proceed as for beef-extract gelatin, but omit beef-extract and peptone. Clarify with white of egg.

Nitrate broth. For routine work add 0.1 per cent KNO_3 to the above formula for beef-extract broth. Routine *nitrate agar* should contain 0.1 per cent KNO_3 added to the ordinary formula

[1] Use a 0.04 per cent solution of brom thymol blue in 95 per cent alcohol.

[2] Use a 0.02 per cent solution of phenol red in 95 per cent alcohol.

for beef-extract agar. Modification of these routine formula is often necessary, as explained below (see p. 130).

Starch agar. Add 0.2 per cent of water-soluble starch to the ordinary beef-extract agar.

Indicator media. Saccharine media with some indicator to show acid production are frequently used. Litmus is the most common indicator, enough of which should be added in saturated aqueous solution to give the medium a distinct blue color (taking care that the litmus solution used is not so alkaline as to alter, appreciably, the reaction of the medium). Litmus, however, does not give accurate results in terms of hydrogen ion concentration; so except for certain special purposes (see p. 130) it is recommended that brom cresol purple be used to detect increase in acidity, and cresol red to detect increase in alkalinity. It is convenient to keep these indicators in concentrated alcoholic solutions of such strength that 1 cc. will be sufficient for each litre of medium. For this purpose a 1.6 per cent solution of either indicator in 95 per cent alcohol is recommended. Brom cresol purple is purple in neutral or alkaline media and is yellow in acid media. Cresol red is yellow at neutrality and in acid media, turning red under alkaline conditions. Under certain circumstances it is desirable to have an indicator that will show a change in either direction from neutrality. Brom thymol blue does this, but is not satisfactory in media because its range is too short to distinguish differences between different kinds of bacteria. Very satisfactory results, however, may be obtained with a mixture of brom cresol purple and cresol red (0.5 cc. each of 1.6 per cent alcoholic solution of the two dyes to the litre of medium.) This mixture changes very slowly from purple to yellow through a long range (from about pH = 8.0 to about pH = 5.0) extending to a considerable distance on both sides of neutrality. By comparing with a blank tube of the neutral medium, it is very easy to detect an increase in either acidity or alkalinity.

Recently certain other combinations of indicators have been recommended for this same purpose. Bronfenbrenner (1919) recommends a combination of china blue with rosolic acid or preferably its sodium salt, and Morishima (1920) a combination

of china blue with phenol red. Both of these combinations are colorless or nearly so at pH = 7.0, turning blue as the reaction becomes acid and red as it becomes alkaline. Rosolic acid has the advantage over phenol red of having a more alkaline range (pH = 7.3 to pH = 9) than phenol red, hence giving the Bronfenbrenner combination a sensitive range from pH = 5 to pH = 9. Rosolic acid is insoluble in water, but it is possible to keep it in concentrated alcoholic solution (as above recommended for brom cresol purple and cresol red) so that only 1 cc. of alcohol is added to a liter of medium. The concentration of china blue in the medium should be 0.0025 per cent, that of rosolic acid or its sodium salt 0.005 per cent, while in combination with china blue a 0.001 per cent solution of phenol red is recommended. Either of these combinations should have distinct advantage over the combination of brom cresol purple with cresol red; but there has been as yet no opportunity to compare them.

Variations of these media. For certain organisms the above formulae are not the best—many pathogenic bacteria, for instance require more peptone than is provided in the above formula for broth, while some organisms are best studied in media of a hydrogen-ion concentration different from that recommended above. In such cases the individual investigator is free to modify the media to suit his own purposes; but whenever other than these routine formulae are used, the fact should be stated on the chart. In employing a reaction other than that of neutrality it is recommended that instead of using the titrimetric method, the reaction be adjusted to some definite shade of brom cresol purple, if a more acid reaction is desired, or of phenol red if it is to be more alkaline.

Optional media. In many laboratories other media than those specifically mentioned on the chart are in general use, such as potato, blood serum, agar stabs, and so forth. Blank spaces are left on the chart for recording characteristics on any optional media.

INVIGORATION OF CULTURES

Provided a medium can be found upon which the organism to be studied grows vigorously, it should always be invigorated before study, even though freshly isolated from its natural habitat. The procedure to employ is as follows:

Prepare duplicate sub-cultures in standard glucose broth, and on standard agar slopes, placing cultures of each at 37° and 25°C. On the basis of the resulting growth the organism falls into one of the following series:

Series I. Organisms which produce good growth (surface growth, distinct turbidity, or heavy precipitate) in twenty-four hours at 37° in glucose broth.

Series II. Organisms which do not produce good growth in twenty-four hours as above, but do in forty-eight hours at 25° in glucose broth.

Series III. Organisms which do not grow well in glucose broth but do produce good growth on the surface of agar in twenty-four hours at 37°.

Series IV. Organisms excluded from the above groups but which produce good growth on the surface of agar in forty-eight hours at 25°.

Record the series number on the chart at the proper place and proceed with the invigoration by inoculating into another tube of glucose broth for organisms of series I and II, or of standard agar for organisms of series III and IV. Incubate this tube at the temperature, and for the time, called for by the series in which it belongs; then transfer from this tube to a third tube and incubate as before. From this third culture make a gelatin or agar plate and incubate at the temperature previously used until colonies of sufficient size for isolation are obtained. Transfer from a typical colony to one or more agar slants and incubate for one day at 37° or for two days at 25° according to the temperature relation of the organism studied.

In case the organism does not produce vigorous growth on either of these media at either temperature, it should be invigorated with any medium and at any temperature known to be

adapted to its growth. Under such circumstances invigorate by the procedure just outlined but using the medium and temperature found most favorable for the organism in question, recording on the chart the method of invigoration adopted. If no conditions are known under which the organism in question produces vigorous growth, it should be studied without preliminary cultivation as soon as possible after isolation from its natural habitat. Such an organism is not likely to give good growth on any ordinary media, and the results of the study called for by the chart will have little significance.

STUDY OF MORPHOLOGY

The routine study of morphology should be from dried preparations, stained with fuchsin, methylen blue, or gentian violet. Preparations to show the vegetative cells should be made, preferably, from agar slant cultures, from a few hours to two days old, according to the rapidity of growth. The medium and temperature used and the age of the culture should be recorded.

Motility. Hanging-drop preparations of young broth or agar cultures should be examined for motility. If motile, microscopic preparations should be made to show the arrangement of the flagella, using any of the ordinary methods of flagella staining with which the student can obtain good success. Even if motility is not observed in hanging-drop, it is wise to attempt a flagella stain, because motile organisms often lose their motility under the conditions of observation. Even negative results from both hanging-drop preparation and flagella stain do not absolutely prove that the organism is immotile.

Presence of spores. Routine examinations for spores should be made on stained, dried preparations from agar slant cultures a week old. Stain with methylen blue. Vegetative forms take the stain, but spores do not. In most cases there will be no trouble in finding spores if the organism produces them. All rather large rods however, (0.8 micron or more in diameter) should be regarded as possible spore-producers, even though microscopic examination does not show spores. Such bacteria should be

mixed with sterile water and heated to 85°C. for ten minutes; if still alive, spores may be regarded as unquestionably present. Also make repeated transfers of the culture onto agar and examine at various ages. A culture of a large rod should not be recorded as a non-spore-former unless all these tests are negative.

Capsules. An organism should not be recorded as having capsules unless they have been actually stained by one of the methods of capsule-staining described in bacteriological text books.

Irregular forms. Forms that differ from the typical shape for the organism (i.e., "involution forms," etc.) such as branching forms, clubs, spindles or filaments should be noted and sketched.

Special stains. Of these the Gram stain has been given particular attention by the committee and at present the Stirling modification is recommended. Further work is being done at present upon an improved method of making this stain, which will be discussed in the next number of the JOURNAL. The Stirling modification is carried out as follows:

Prepare gentian violet solution by grinding 5 grams in 10 cc. of 95 per cent alcohol in a mortar. Add 2 cc. anilin oil, distilled water 88 cc. Filter.

Iodine solution is as usual: 1 gram iodine, 2 grams potassium iodide, 300 cc. water.

The procedure recommended in the 1918 report was as follows: one minute in stain; wash in water; one minute in iodine solution; wash in water; two minutes in absolute alcohol; wash in water; thirty seconds in counter stain (10 cc. saturated alcoholic safranin in 90 cc. water). This procedure gives very good results; but recent work undertaken in the army cantonments shows that equally good results can be obtained by the following rapid method: one to five seconds in stain; wash in water; five to ten seconds in iodine solution; wash in water; ten to twenty seconds in absolute alcohol; wash in water; five to ten seconds in counterstain.

We are informed that the Stirling solution of gentian violet can be made more stable by mixing normal hydrochloric acid with the anilin oil before dissolving in water. (Add 0.5 cc. normal HCl to 2 cc. anilin oil; dissolve in 88 cc. water and filter;

mix with 10 cc. of alcoholic gentian violet prepared as above.) The committee has not yet tested out this procedure.

Sketches. Drawings of all the morphological characteristics should be made on the blank spaces on the chart to the right of the descriptions. Both typical and atypical forms should be sketched, using care to designate which are typical.

CULTURAL CHARACTERISTICS

Cultures for the study of cultural characteristics should be incubated at 37°C. in case of organisms of series I and III, and at 25° in case of organisms of series II and IV, except that gelatin cultures should be incubated at 20°. Room temperature may be used in place of 25° at certain seasons of the year; but if a minimum thermometer shows that the temperature falls below 22° during the course of the work, note should be made of the fact. On the day when good growth first appears the proper descriptive terms on the card should be underlined; after subsequent study, the changes should be noted in the space provided, and sketches of the different stages should be made.

PHYSIOLOGY

Liquefaction of gelatin. Old method. The method in most common use is to hold gelatin stab cultures six weeks at 20°C. Plain gelatin should be used.

Provisional method. It is recommended that the following method proposed by Rothberg (1917) be put in provisional use until experience shows its value. It is designed to distinguish "true liquefiers" (organisms producing ecto-enzymes) from the organisms that produce endo-enzymes of proteolytic action that are released from the cell after death and cause liquefaction of the gelatin if incubated for the long period mentioned above. The method is to give the organism a preliminary cultivation for eighteen to forty-eight hours (according to its rapidity of growth) in a 1 per cent solution of gelatin at 25° or 37° according to its temperature relations; then inoculate on surface of gelatin in test tube and incubate fifteen days at 20°.

Relation to free oxygen. Provisional method. Determine by noting the presence or absence of growth in open and closed arm, respectively, of fermentation tubes containing glucose broth. Care must be taken to use fermentation tubes from which the dissolved oxygen has been recently driven off by heating. In case of gas production, this test is of comparatively little value, because bubbles of gas may carry the sediment up with them; hence if an organism produces gas from glucose, the test should, if possible, be made in the presence of some other sugar which it attacks (acidifies) without gas-formation. It must be remembered, however, that even anaerobes do not grow in the absence of free oxygen except in the presence of a chemical substance (such as carbohydrate) which they are able to reduce and use as a source of oxygen.

Fermentation of sugars and glycerol. This is normally to be studied in fermentation tubes. Ordinarily use beef-extract broth containing 1 per cent of the substance investigated; but if the organism does not grow well in such broth and some medium is known in which it does grow well, the latter may be used. Generally speaking, organisms of series I and II should be studied in broth, organisms of series III and IV in some other medium. Incubate organisms of series I and III at 37°, organisms of series II and IV at 25°. Test ordinarily on 1st, 3rd, and 7th days, although the best days for testing will depend upon the rapidity of growth of the culture. Hence on the chart, although space is given for recording reaction on three separate days, blanks are left for the individual student to fill in with the days upon which the tests are actually made. Inoculations should always be made at least in triplicate.

To test for acid, it is recommended that in place of the illogical titrimetric method, determinations of hydrogen-ion concentration be made by the colorimetric method described by Clark and Lubs (1917 a). In accurate research work the exact shade of the indicator should be compared with that obtained in standard "buffer" solutions, and results recorded in terms of pH. In laboratories where these standard solutions cannot be obtained, it is better to record results simply as + or −, according to the

reaction of the culture to litmus, than to use the titration method. Under such conditions it is possible, however, to obtain a rough idea of the hydrogen-ion concentration by the use of Clark and

TABLE 1

Degrees of acidity easily recognized in clear media

ACIDITY	INDICATOR REACTIONS	APPROXIMATE pH-VALUE
"Neutral"	Blue or green to brom thymol blue*	Over 6.2
"Weak"	Yellow to brom thymol blue Purple to brom cresol purple*	5.2–6.0
"Moderate"	Yellow to brom cresol purple Orange to methyl red†	4.6–5.0
"Strong"	Maximum red to methyl red Blue or green to brom phenol blue*	3.2–4.4
"Very strong"	Yellow to brom phenol blue	Under 3.0

* Use a 0.04 per cent alcoholic solution.
† Use a 0.02 per cent alcoholic solution.

TABLE 2

Degrees of acidity easily recognized in milk

ACIDITY	INDICATOR REACTION, ETC.	APPROXIMATE pH-VALUE
"Neutral"	Same color with brom cresol purple* as sterile milk; i.e., blue to gray green	6.2–6.8
"Weak"	Color with brom cresol purple lighter than in sterile milk; i.e., gray-green to greenish yellow	5.2–6.0
"Moderate"	Yellow with brom cresol purple. Not curdled	4.7–6.0
"Strong"	Curdled. Blue or green to brom phenol blue*	3.2–4.6
"Very strong"	Yellow to brom phenol blue	Under 3.0

* Use a 0.04 per cent alcoholic solution.

Lubs' series of indicators without making accurate determinations of pH. Four different degrees of acidity can be easily distinguished by this simple method in sugar broth with an initial reaction of neutrality. The indicator reactions for these differ-

ent degrees of acidity are listed in table 1, together with the approximate range of pH to which each corresponds. In the absence of accurate determinations, these degrees of acidity may be recorded by the indefinite terms, "weak," "moderate," "strong and "very strong," or by the symbols +, ++, +++, and ++++. If the student desires to record increase in alkalinity in the same table on the chart, he can use the symbol 0 for neutrality and − for an alkaline reaction.

Gas production is ordinarily measured in percentage of gas in the closed arm, and the ratio of $H:CO_2$ by means of absorption with NaOH, using the methods described in laboratory manuals (filling open arm with 4 per cent NaOH, allowing gas to enter open arm, shaking and returning gas to closed arm). As this method is far from accurate, it is recommended for provisional use only.

The fermentation test is ordinarily of no significance for organisms of series III and IV because of their poor growth in broth. Sometimes these organisms can be studied in some other liquid medium in which they do give good growth; but generally it is preferable to use agar slants. In such a case, use a sugar agar containing brom cresol purple, china blue, or a mixture of indicators as suggested on page 129. Increase in acidity can be detected by fading of the purple color of the brom cresol purple or by the appearance of blue if china blue is used. Gas-production can usually be detected in agar cultures by the presence of cracks and air bubbles, but as a test for gas, agar slants are not as reliable as fermentation tubes.

A more detailed discussion of hydrogen-ion concentration and of methods for determining acid-production is given in the 1918 report of this committee, copies of which can be obtained from the chairman.

Milk. Acid production in milk can be detected by adding brom cresol purple to the culture and comparing with the color obtained by adding the same proportionate quantity of indicator to sterile milk. (Brom thymol blue does not give satisfactory results in milk.) Four degrees of acidity that can be simply recognized in milk are listed in table 2. They correspond closely

to those listed in table 1, differing only in that brom cresol purple is used instead of brom thymol blue to show "neutrality" and that the curdling point (pH = 4.7) is used to separate between "moderate" and "strong" acidity instead of the less definite point of maximum red to methyl red. The same methods of expression used in recording acidity in clear media should be used in recording that of milk.

Litmus milk often gives valuable information, showing not only the production of acid, but also decolorization of the litmus by organisms that are able to reduce it. More accurate results as to acidity can be obtained by using brom cresol purple, as shown by Clark and Lubs (1917 b). This indicator, however, does not show the reduction phenomena which are sometimes of diagnostic value in litmus milk cultures; its substitution for litmus is not, therefore, always to be recommended.

Reduction of nitrates. The following procedure is recommended: Inoculate first into nitrate broth and onto slants of nitrate agar, the media having the composition given on p. 128. Test the cultures on various days as indicated on the chart. On these days examine first for gas as shown by foam in the broth or by cracks in the agar. Then test for nitrite with the following reagents:

(1) Dissolve 8 grams sulphanilic acid in 1 litre of 5 N acetic acid (1 part glacial acetic acid to 2.5 parts of water), or in 1 litre of dilute sulphuric acid (1 part concentrated acid to 20 parts water).

(2) Dissolve 5 grams α-naphthylamine in 1 litre of 5 N acetic acid or of very dilute sulphuric acid (1 part concentrated acid to 125 parts water).

Put a few drops of each of these reagents in each broth culture to be tested, and on the surface of each agar slant. A distinct pink or red in the broth or agar indicates the presence of nitrite. It is well to test a sterile check which has been kept under the same conditions, to guard against errors due to absorption of nitrite from the air. Presence of nitrite or of gas shows the nitrate to have been reduced. A negative result does not prove that the organism is unable to reduce nitrates; in such a case further study is necessary, as follows:

In case the fault seems to lie in poor growth, search should be made for a nitrate medium in which the organism in question does make good growth by means of the following modifications: increasing or decreasing the amount of peptone; altering the reaction; adding some readily available carbohydrate. Presence of nitrite or gas in any nitrate medium whatever should be recorded as nitrate-reduction. Unless the routine formula is used, the exact composition of the medium must always be given.

If the organism grows well and yet produces no nitrite or gas, the determination must be recorded as doubtful unless the organism can grow well in some synthetic medium containing no nitrogen except nitrate. It is recommended that such an organism be tested in a medium containing small quantities of phosphate, calcium, chlorine, etc., with KNO_3 as a source of nitrogen and sucrose as a source of energy and of carbon.[3] Such a medium generally allows good growth with an organism capable of utilizing nitrate and sucrose. Unfortunately neither glucose nor lactose can be used in this medium as a source of carbon and energy, for the ordinary "c.p." preparations of these sugars contain much ammonia. If the organism in question grows (even but slightly) on a synthetic medium of this sort, it should be tested for nitrite by the usual method and for ammonia by means of Nessler's reagent (comparing with an uninoculated tube as a check). The presence of nitrite, of ammonia (i.e., a more pronounced ammonia reaction than in check tube), or of gas indicates nitrate-reduction.

The production of gas (free N) from nitrate is not a very common one; but a considerable number of soil organisms have this power, and one should be on the lookout for it in studying soil bacteria. The agar slant test is ordinarily a sufficiently delicate test; but, if liquid media are used, more reliable results may be obtained by the use of fermentation tubes.

Chromogenesis. Color production should be recorded if observed in broth, on beef-extract agar, gelatin or potato, or if

[3] An illustration of such a medium which has proved satisfactory for some bacteria is: K_2HPO_4, 0.5 gram, $CaCl_2$, 0.5 gram, KNO_3, 1 gram, sucrose 10 grams, agar 12 grams, water 1000 cc.

noticed to a striking extent on any other medium. In the group number, the point devoted to chromogenesis refers to the color produced on beef-extract agar.

Diastatic action on starch. Provisional method. Use beef-extract agar containing 0.2 per cent of soluble starch. Pour into a petri dish, and after hardening make a streak inoculation on its surface. Incubate at 37° for organisms of series I and III, at 25° for organisms of series II and IV. Determinations for the group number shall be based upon results obtained on the seventh day. To make the test, flood the surface of the petri dishes with a saturated solution of iodine in 50 per cent alcohol. The breadth of the clear zone outside of the area of growth indicates the extent of diastatic action. If over 2 mm. in width on the seventh day it shall be recorded as "strong;" if under 2 mm. as "feeble;" if no clear zone is present, as "absent."

This method requires some modification, e.g., reducing the amount of peptone in the medium, for organisms that grow so rapidly as to cover the entire surface of the plate in seven days, thus leaving no room for a clear zone outside.

The group-number is a brief means of recording the salient features of the organism. It is primarily a summary of the physiological characteristics just discussed. As each of the determinations is made, the proper figure for that place in the group number is to be checked or underscored. After completing the determinations, the entire group number is to be written in at the place left for it on the chart. The genus symbol should precede the group number. The present group number, adopted by the Society in 1907, was intended for use with the generic names of Migula. As Migula's genera are not in such general use today as they were ten years ago, a revision of the group number on some other basis is now being undertaken by the committee, and will be discussed in Part II of this report.

Brief characterization. On the right hand margin of page one of the chart is a place for recording by a + or − sign other important characteristics of the organism (primarily cultural)

not included in the group number. This margin together with the group number constitute a brief characterization of the organism—a summary of the tests outlined above.

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Glossary of Terms Used on the Chart

Adherent, Applied to sporangium wall, indicates that remnants of sporangium remain attached to endospore for some time.

Aerobic, growing in the presence of free oxygen; strictly **aerobic**, growing *only* in the presence of free oxygen.

Amorphous, without visible differentiation in structure.

Anaerobic, growing in the absence of free oxygen; **strictly anaerobic,** growing *only* in the presence of free oxygen; **facultative anaerobic,** growing both in presence and in absence of free oxygen.

Arborescent, branched, tree-like growth.

Beaded, (in stab or stroke culture) disjointed or semi-confluent colonies along the line of inoculation.

Bipolar, at both poles or ends of the bacterial cell.

Brittle, growth dry, friable under the platinum needle.

Butyrous, growth of butter-like consistency.

Chains, four or more bacterial cells attached end to end.

Chromogenesis, the production of color.

Ciliate, having fine, hair-like extensions, resembling cilia, sometimes not visible to the naked eye.

Clavate, club-shaped.

Coagulation, the separation of casein from whey in milk.

Contoured, an irregular, smoothly undulating surface, like that of a relief map.

Convex, surface the segment of a sphere.

Crateriform, a saucer-shaped liquefaction of the medium.

Cuneate, wedge-shaped.

Curled, composed of parallel chains in wavy strands, as in anthrax colonies.
Diastatic action, conversion of starch into simpler carbohydrates, such as dextrins or sugars, by means of diastase.
Echinulate, a growth along line of inoculation with toothed or pointed margins.
Effuse, growth thin, veily, unusually spreading.
Endospores, thick-walled spores formed within the bacterial cell; i.e., typical bacterial spores like those of *B. anthracis* or *B. subtilis*.
Entire, with an even margin.
Erose, border irregularly toothed.
Filaments, applied to morphology of bacteria, refers to thread-like forms, generally unsegmented; if segmented, to be distinguished from chains (q.v.) by the absence of constrictions between the segments.
Filamentous, growth composed of long, irregularly placed or interwoven threads.
Filiform, in stroke or stab cultures, a uniform growth along line of inoculation.
Flocculent, containing small adherent masses of bacteria of various shapes floating in the culture fluid.
Fluorescent, having one color by transmitted light and another by reflected light.
Granular, composed of small granules.
Infundibuliform, form of a funnel or inverted cone.
Iridescent, exhibiting changing rainbow colors in reflected light.
Lobate, having the margin deeply undulate, producing lobes (see *undulate*).
Luminous, glowing in the dark, phosphorescent.
Maximum temperature, temperature above which growth does not take place.
Membranous, growth thin, coherent, like a membrane.
Minimum temperature, temperature below which growth does not take place.
Mycelioid, colonies having the radiately filamentous appearance of mold colonies.
Napiform, liquefaction in form of a turnip.
Opalescent, resembling the color of an opal.
Optimum temperature, temperature at which growth is most rapid.
Papillate, growth beset with small nipple-like processes.
Pellicle, bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.
Peptonization, rendering curdled milk soluble by the action of peptonizing enzymes.
Peritrichiate, covered with flagella over the entire surface.
Persistent, lasting many weeks or months.
Plumose, a fleecy or feathery growth.
Polar, at the end or pole of the bacterial cell.
Pulvinate, decidedly convex, in the form of a cushion.
Punctiform, very small, but visible to naked eye; under 1 mm. in diameter.
Radiate, showing ray-structure.
Raised, growth thick, with abrupt or terraced edges.
Reduction, removing oxygen from a chemical compound. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen, and to the decolorization of litmus.
Rhizoid, growth of an irregular branched or root-like character, as in *B. mycoides*.
Ring, growth at the upper margin of a liquid culture, adhering to the glass.
Rapid, developing in twenty-four to forty-eight hours.

Rugose, wrinkled.
Saccate, liquefaction in form of an elongated sac, tubular, cylindrical.
Slow, requiring five or six days for development.
Spindled, larger at the middle than at the ends. Applied to sporangia, refers to the forms frequently called **clostridia.**
Sporangia, cells containing endospores.
Spreading, growth extending much beyond the line of inoculation, i.e., several millimeters or more.
Stratiform, liquefying to the walls of the tube at the top and then proceeding downwards horizontally.
Transient, lasting a few days.
Truncate, ends abrupt, square.
Turbid, cloudy with flocculent particles; i.e., cloudy plus flocculence.
Umbonate, having a button-like, raised center.
Undulate, border wavy, with shallow sinuses.
Viscid, growth follows the needle when touched and withdrawn; sediment on shaking rises as a coherent swirl.

NOTES ON THE CLASSIFICATION OF THE WHITE AND ORANGE STAPHYLOCOCCI

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INTRODUCTION

The white and orange cocci of the skin were first isolated in pure culture by Rosenbach (1884) and described as var. *aureus* and var. *albus* of a species which he named *Staphylococcus pyogenes*. In later years such systematic bacteriologists as Migula (1900) and Chester (1901) discarded the generic name suggested by Rosenbach and grouped these organisms along with the large series of yellow and red cocci under the genus, *Micrococcus*. For some time all thought of any important differences correlated with variations in pigment production appeared to be abandoned; and yellow, as well as white and orange, cocci found upon the skin were commonly classed by medical observers as varieties of a single species.

In 1908 the Winslows showed that, in the case of the yellow and red chromogens at least, the type of pigment production was associated with other characteristics of fundamental systematic importance. They made it clear that the white and orange cocci belong to a series of cocci, including the streptococci and diplococci, which are essentially parasitic in nature, Gram positive and active in fermentative power, while the yellow and red forms (including the sarcinae) are normally found in environments outside the human body, are Gram-negative and exhibit a much more restricted fermentative activity.

The Gram-positive, acid-forming parasitic cocci, which occur in irregular growth masses rather than in pairs or chains, and which produce a fairly abundant growth on media, were again

subdivided by the Winslows into two genera—*Aurococcus* including the orange pigment producers, and *Albococcus* including the forms which produce a more abundant growth, of porcelain white color. Buchanan (1915) has pointed out that in spite of the neglect of the generic term *Staphylococcus* by recent systematic writers, the name has perfectly valid standing according to the accepted rules of biological nomenclature and should be used in place of *Aurococcus* for the orange pigment formers. The Committee on Classification (1917) of the Society of American Bacteriologists accepted this view and in its first report recognized the genera, *Staphylococcus* (orange pigment formers) and *Albococcus* (white pigment formers).

In the discussions which followed the report of the Committee on Classification the more fundamental question was raised as to whether—aside from any questions of terminology—the separation of the orange and white staphylococci into two distinct genera was justified.

The Winslows based the distinction primarily on the difference in pigment production, less heavy growth on media and more vigorous liquefaction of gelatin by the orange forms. Both orange and white groups included some non-liquefiers but among those which did produce liquefaction the orange forms were twice as active. Earlier results of Dudgeon (1908) were cited as suggesting that the orange chromogens differ from the white forms in exerting a more powerful reducing action on neutral red and a more active fermentation of mannitol, glycerol and raffinose. Dudgeon, himself, however, finally concluded that the white and orange staphylococci were all varieties of a single species. The Winslows reviewed the results obtained by various other observers in regard to the variability of chromogenic power among the staphylococci but finally, in spite of such observations, recognized the white and orange forms as constituting different genera. Kligler (1913) from a study of the strains of staphylococci in the collection of the American Museum of Natural History concluded that the difference in rate of gelatin liquefaction between the white and orange cocci was a valid one.

Both these American systematic studies of the staphylococci were based on a rather small series of strains. The Winslows had 181 orange forms in their series, of which 126 were liquefiers, but only 23 white forms including 14 liquefiers. Kligler studied only 15 orange and 12 white strains. In order to throw further light on the relationships of these organisms, it seemed worth while to collect a larger series of strains and to submit them to a more exhaustive series of quantitative tests. This was the purpose of the present study.

SOURCE OF CULTURES

Our aim in collecting the cultures for this investigation was to obtain 100 strains, each, of white and orange cocci, of which about half should be from the human body and about half from air, dust, water and other environmental sources.

We finally obtained a total of 185 strains, of which 5 were discarded for reasons to be noted later; and our complete studies were conducted on 180 different strains. Of these, 104 were from pathological conditions in men and animals, 22 were isolated from the hands and 54 were isolated from air, dust or water.

The 104 pathogenic strains were courteously furnished for our use by Parke, Davis and Company, H. K. Mulford Company, The Abbott Laboratories and the Lederle Laboratories, and they came from a widely diversified series of conditions of which the following were the most important; abscesses, acne, boils, cellulitis, conjunctivitis, coryza, endometritis, furunculosis, gonorrhea, impetigo, osteomyelitis, otitis media, pharyngitis, pyorrhea, prostatitis, sarcoma, septicemia, sinus infection, tonsillitis, tuberculosis, ulcers, urethritis, whooping cough and wound infections in man; abscesses, distempers, equine influenza, mange, nasal discharge and septicemia in the horse. Whether the organisms sent to us bore any etiological relation to these diseased conditions is of course uncertain, but they were in all cases isolated from tissues in which an active disease process was in progress.

The 22 cultures isolated from the hands were obtained by rinsing the surface of the hands in sterile water and plating on glucose agar, from which characteristic white and orange colonies were fished.

Of the 54 strains which came from sources other than the human body 39 were obtained from the air by exposing glucose agar plates in the offices and workrooms of the American Museum, in the New York Subway, and in the streets and parks of New York City. Thirteen were isolated from dust collected in the same general localities, one strain was isolated from a sample of butter and one from water.

In analyzing our results we have divided our strains from the standpoint of source into these three main groups, 104 isolated from pathogenic conditions, 22 from the hands and 54 from sources outside the human body.

CHROMOGENESIS

The chromogenic power of our 185 strains was determined as follows: Each strain was cultivated on a nutrient agar streak for fourteen days at 20°C. A portion of the growth was then spread with a platinum loop over white paper (Whatman No. 2) and allowed to dry in the air. The hue and tint were then matched against the frontispiece of the Systematic Relationships of the Coccaceae (by C.-E.A. and A. R. Winslow). The determinations of chromogenesis were made for the whole series on two different occasions about six months apart.

Four strains originally isolated as orange pigment producers proved on examination to belong to the group of the red chromogenic cocci (*Rhodococcus*) and were excluded from subsequent consideration. One strain gave inconsistent results on the two series of tests being recorded as orange on the first occasion and white on the second. This strain was also excluded from our series leaving 180 strains for detailed study.

These 180 strains divided themselves naturally into two very clearly marked groups, as did the cocci studied by the Winslows. One hundred of them were of the *Albococcus* type giving a pigment which could be matched in the Light Lemon Yellow,

Light Cadmium Yellow and Medium Cadmium Yellow of the Winslow's chart, usually in the three lightest chromas. The other 80 were of the *Aurococcus* type, their pigment being matched by the Orange Yellow and Cadmium Orange hues, usually in the darker chromas.

So far as pigment production alone is concerned, these two types are clearly and definitely to be distinguished from each other.

Of the 104 strains isolated from pathological conditions 53 were of the white and 51 of the orange type. Of the cultures from the hands 16 were white and 6 orange and of the air and dust strains, 31 were white and 23 orange. No correlation appears, therefore, to exist between chromogenesis and habitat.

GRAM STAIN

The Gram stain was made by the method described in the Systematic Relationships of the Coccaceae. Agar cultures which had been incubated for twenty-four to forty-eight hours at 20° and 37°C. respectively were treated with anilin-oil-gentian violet for one and one-half minutes; with Gram's solution for one and one-half minutes; with 95 per cent alcohol for three minutes; and counterstained with Bismarck Brown for one-half minute. Altogether we repeated the whole series five separate times.

The cocci exhibit a somewhat variable reaction to the Gram stain but of our 180 strains 136 were consistently positive on all five occasions, 39 were generally positive but gave negative results on 1 or 2 out of the five trials and 5 were consistently negative. These 5 strains had no other special characteristics in common except that all were gelatin liquefiers.

LIQUEFACTION OF GELATIN

The property of gelatin liquefaction was studied in the following manner. Each strain was first grown for twenty-four hours at 37°C. in nutrient broth containing 1 per cent gelatin and one loopful of this culture was then spread over the surface

of ordinary nutrient gelatin in tubes of $\frac{5}{8}$ inch diameter. These gelatin tubes were incubated at 20°C. for thirty days and the depth of the gelatin liquefied was recorded at frequent intervals by measuring down to the surface of the solid portion from a line drawn with a glass pencil around the tube at the original level of the medium.

Of the total of 180 strains studied, 101 liquefied gelatin to some degree within thirty days. Of 100 white strains, 47 (47 per cent) were liquefiers; of 80 orange strains 54 or 67 per cent were liquefiers, indicating a slightly higher tendency to attack gelatin within this color group as noted in the Systematic Relationships of the Coccaceae. It was also suggested by the

TABLE 1

Average depth of gelatin liquefaction in centimeters after various intervals (at 20°C.)

	DAYS											
	1	3	5	7	9	11	14	16	18	21	25	30
	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.
Orange forms	0	0.1	0.2	0.6	1.0	1.2	1.2	1.7	2.0	2.4	2.8	3.1
White forms	0	0.1	0.2	0.2	0.6	1.0	1.1	1.4	1.5	1.8	2.1	2.5

Winslows that the orange forms when they do liquefy act more rapidly and more vigorously than the white cocci. We find that there is indeed a slight difference of this kind as indicated by table 1, which, however, shows the differences observed by us to be much slighter than those recorded in earlier investigations. The Winslows report an average liquefaction after thirty days of 2.2 cm. for the orange forms and 1.1 cm. for the white strains; while Kligler cites figures of 3.5 cm. for the orange and 1.4 cm. for the white forms. The Winslows studied only 16 white liquefying strains and Kligler only 3; and their results are probably not typical. The fact that the absolute values recorded in the present investigation are so much higher than those reported by the Winslows may in part be attributed to the preliminary cultivation in gelatin broth; but it seems clear from the fairly large series of strains studied by us that the difference in rate of gelatin liquefaction between the white and orange staphylococci is only a relative and a rather slight one.

FERMENTATION OF CARBOHYDRATE MEDIA

In view of the comparatvely slight difference in gelatinolytic power between the white and orange cocci, it seemed important to study as many other biochemical properties as possible in order to determine whether the two chromogenic types were really deserving of generic rank; and in view of the important light thrown by the study of fermentative reactions upon the systematic relationships of the colon-typhoid group we have devoted considerable attention to this point. Very little previous work has been done on the power of the staphylococci to ferment various carbohydrate media. It is well known of course that they usually attack the more familiar sugars with the formation of acid, but no gas. Gordon (1906) studied the action of the white staphylococci on lactose, maltose, glycerol and mannitol. Dudgeon (1908) used a considerable series of carbohydrates but his work was not quantitative. The Winslows studied glucose and lactose only while Kligler (1913) added sucrose. In both cases generally positive results were reported.

In our own study we have used not only glucose, lactose and sucrose but also maltose, raffinose, mannitol, dulcitol, salicin and inulin. Two different media were employed in this study, the dehydrated bacto nutrient broth prepared by the Digestive Ferments Company and the peptone medium of Clark and Lubs.

The dehydrated medium contained 3 parts of bacto beef extract and 5 parts of bacto peptone and when dissolved (8 grams to a liter of distilled water) and sterilized for twenty minutes at 15 pounds, it had a pH value between 6.7 and 6.8. The peptone medium contained 0.5 per cent K_2HPO_4 and 0.5 per cent Witte's peptone; and was adjusted to a pH value of 7.4.

To each medium was added 2.7 per cent of a 0.04 per cent alcoholic solution of bromcresol purple before sterilization (as suggested by Bronfenbrenner, 1918) and 0.5 per cent of sterile carbohydrate after sterilization. The pH value was determined after 1, 3, 5 and 7 days of incubation at 30°C. by matching the tubes against the standards of Clark and Lubs (1917).

The bromcresol purple indicator gives a reasonably sharp color change between pH values of 5.2 and 6.8 but above 6.8 the color change is slight and the adjustment of the final endpoint of the peptone medium was therefore somewhat uncertain. It was always, however, over 6.8.

In general we found that glucose, lactose, maltose and sucrose were fermented by a large majority of the cultures studied while raffinose, mannitol, dulcitol, salicin and inulin were but rarely attacked. The analysis of the results was materially complicated, however, by the presence of three distinct modal points of acidity and by the variation between the results obtained in the two different media studied.

In figure 1 we have presented the distribution of the pH values recorded after seven days of incubation in the two types of broth and with the four carbohydrates which were most readily attacked. It will be noted that in nearly all the curves there are three more or less distinct modes, one at a pH value between 5.0 and 5.3, indicating vigorous fermentation, a second at a pH figure between 6.4 and 6.9, and a third at 7.4. In the succeeding discussion we shall designate these three reactions as acid (+), neutral (±), and alkaline (−). A neutral reaction indicates no change in the case of the dehydrated medium (which started at a pH of 6.8) while it implies a slight production of acid in the case of the peptone medium which started at 7.4. A neutral reaction in the dehydrated medium and an alkaline reaction in the peptone medium alike indicate no change in reaction while an alkaline reaction in the dehydrated medium implies a real decrease in acidity.

In general, as might be expected, the dehydrated broth with its slightly lower initial alkalinity, showed a higher final acidity than did the peptone broth with its slightly higher initial alkalinity. Out of 180 strains tested in glucose, 116 were ultimately acid in both media, 29 were acid in dehydrated and neutral in peptone broth, 8 were acid in dehydrated and alkaline (indicating no change) in peptone broth, 12 were neutral in dehydrated and alkaline in peptone broth (indicating no change in either case) and 16 were alkaline in both (indicating slight alkali pro-

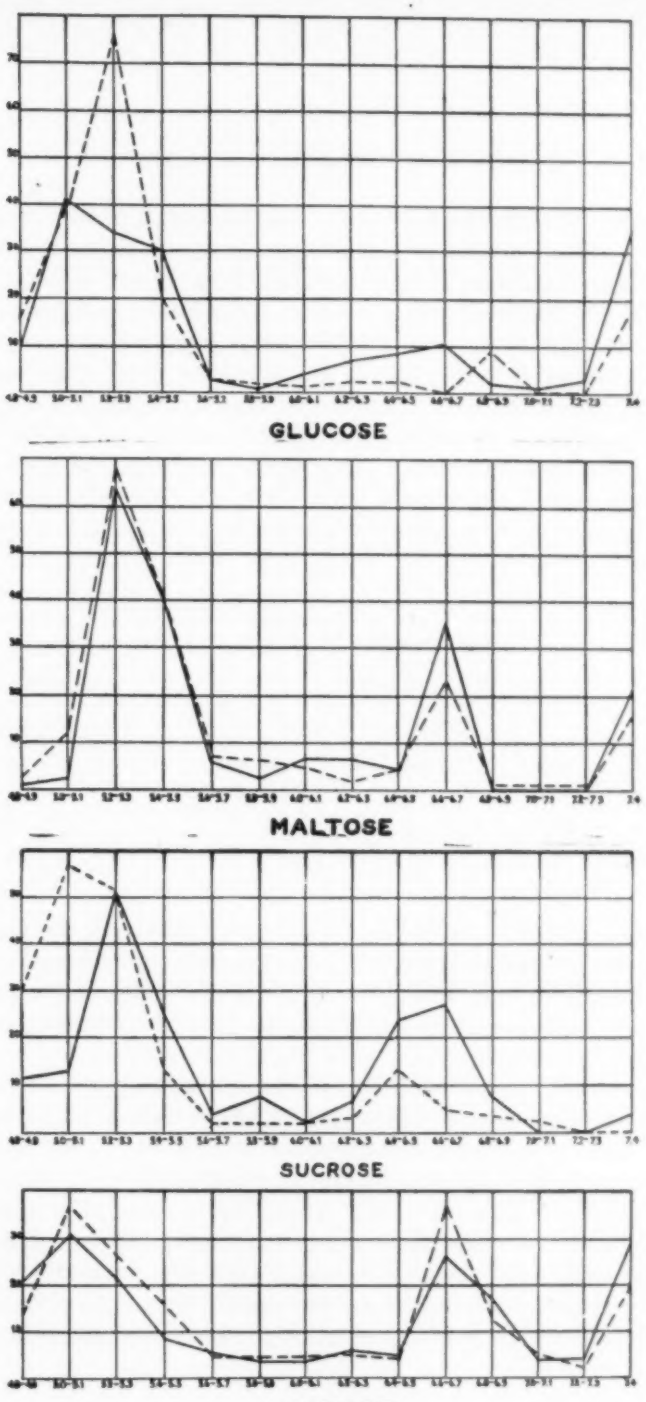

FIG. 1. ACID PRODUCTION BY STAPHYLOCOCCI ORDINATES, PER CENT OF STRAINS, ABSCISSAE P_H VALUES

duction in dehydrated broth). The average reactions for these groups after various periods of incubation are indicated below.

The 116 cultures which ultimately became acid in both cases (class A) changed more slowly in the peptone medium, either because of its greater initial alkalinity, greater buffer action, or lower nutritive value, but by the fifth day at 30° they reached about the same point. Members of class B changed more slowly in the dehydrated medium than did those of class A but reached the same final end point, while in the peptone medium they never passed beyond the neutral point. Class C behaved like class B in the dehydrated medium, but in the peptone medium showed no

TABLE 2

Average pH *values in dehydrated and peptone media*

CLASS	DEHYDRATED MEDIUM					PEPTONE MEDIUM				
	Final reaction	pH value after				Final reaction	pH value after			
		1 day	3 days	5 days	7 days		1 day	3 days	5 days	7 days
A	Acid	6.1	5.3	5.2	5.2	Acid	7.2	5.8	6.3	5.2
B	Acid	6.4	5.9	5.4	5.3	Neutral	7.3	7.0	6.6	6.4
C	Acid	6.9	5.7	5.3	5.2	Alkaline	7.4	7.4	7.3	7.3
D	Neutral	7.2	6.9	6.7	6.7	Alkaline	7.4	7.4	7.1	7.3
E	Alkaline	7.3	7.4	7.4	7.4	Alkaline	7.3	7.4	7.4	7.4

change. Class D and class E showed no change in the peptone medium but slight, transitory or permanent, production of alkali in the dehydrated medium. In view of the difficulty in making accurate readings over 6.8 these last two groups may best be considered as one.

We may therefore consider the strains studied by us as divided into two main groups, Classes A, B and C which strongly acidify the dehydrated broth and classes D and E which fail to form acid in either medium. The first group, however, includes 116 strains which show an equally strong acid production in the peptone broth, 29 which show a slower acid production in the peptone broth and 8 which show no change in peptone broth.

It appears evident that we are dealing here with a group of organisms which generally attacks glucose but which contains a

considerable proportion of strains that fail to do so if the composition of the medium is not altogether favorable.

The distribution of results in broth containing maltose, sucrose or lactose is generally similar except that the differential effect of the peptone broth seems to be less marked in the case of maltose and lactose.

Throughout all the tests, however, the dehydrated broth is characterized by a higher acidity than the peptone medium.

TABLE 3

Final reactions (after seven days at 30°)

CARBOHYDRATE	PERCENTAGE OF CULTURES IN EACH CLASS					
	Peptone broth			Dehydrated broth		
	Acid	Neutral	Alkaline	Acid	Neutral	Alkaline
Glucose	68	15	16	84	7	9
Maltose	63	26	10	75	16	8
Sucrose	61	35	3	83	14	2
Lactose	49	33	18	54	33	12
Salicin	5	89	5			
Inulin	1	91	7			
Raffinose	1	78	21			
Dulcitol	0	92	8			
Mannitol	0	87	12			

The proportion of acid, neutral and alkaline reactions for each of the carbohydrates studied is indicated in the table above. Raffinose, mannitol, dulcitol, salicin and inulin were studied only in the peptone medium.

It may be of interest to compare with the results presented in table 3 the data obtained by Dudgeon (1908) using the change in the color of litmus as the sole measure of acid production. We have calculated the percentage of positive results obtained by him for his two chief groups of 46 orange strains and 71 white strains in table 4.

Glucose, maltose, sucrose and lactose appear most readily fermentable in either case, but the use of litmus (which is a highly inaccurate indicator), with no study of the progressive change in reaction, led Dudgeon to report a large number of positive results

with mannitol, raffinose, inulin and salicin (and perhaps with glycerol and erythritol) which as our observations suggest were probably erroneous.

From our detailed quantitative studies with the new indicators it seems evident that salicin, inulin, raffinose, dulcitol and mannitol are attacked by the staphylococci so rarely as to be of no serious diagnostic value; that glucose, maltose and sucrose are most readily attacked and with about equal frequency, and that lactose is slightly, though distinctly, less available than glucose, maltose and sucrose. It is of interest to note that the indicated metabolic gradient is different from that which occurs in the

TABLE 4

Fermentation results reported by Dudgeon (1908) on basis of change in color of litmus

MEDIUM	ORANGE	WHITE
	per cent positive	*per cent positive*
Glucose	100	100
Lactose	100	96
Mannitol	89	49
Maltose	100	96
Glycerol	73	54
Sucrose	98	93
Raffinose	64	42
Erythritol	40	21
Salicin	46	41
Inulin	33	28

colon group or among the streptococci. In both the latter groups lactose is more readily attacked than sucrose and among the colon bacilli, at least, sucrose and raffinose (the two ketonic sugars) are fermented with equal frequency. Among the staphylococci the fermentative processes involved must be distinctly different since sucrose is more readily fermented than lactose while raffinose is not attacked at all.

The action of the staphylococci upon glucose, maltose, sucrose, and lactose would seem to offer a possible basis for classification, although the marked differences due to the effect of the medium indicated in figure 1 would suggest that the use of this property as a differential test might prove of doubtful value.

For simplicity of analysis we have used in the following comparison only the results obtained in dehydrated broth, which yielded the clearest differentiation between fermenting and non-fermenting forms, and have assumed that in this medium all pH values above 6.0 indicated lack of fermentative power.

Of the 180 cultures studied 90 or 50 per cent produced a distinct acid reaction (pH 5.9 or below) in all four sugars; 41 or 23 per cent produced an acid reaction in glucose, maltose and sucrose but not in lactose; 11 or 6 per cent fermented glucose, maltose and sucrose but not lactose; 11 or 6 per cent fermented glucose and sucrose only; 23 or 13 per cent attacked none of the sugars; while 15 or 8 per cent showed special variations not fitting into either group (glucose-lactose-sucrose, glucose-lactose-maltose, maltose-sucrose, glucose-lactose, lactose-sucrose, glucose alone, sucrose alone, maltose alone).

In view of the marked variations shown in figure 1 it would seem unsafe to lay stress upon any of the smaller groups indicated by this analysis; and for comparison with other characteristics of the organisms in question we have therefore divided them into three main groups, group I those fermenting all four sugars; group II those fermenting glucose, maltose and sucrose, but not lactose; and group III, including all the rest of the strains.

Group III is a highly heterogeneous agglomeration; but the forms which fail to ferment lactose (group II) seem to constitute a fairly well defined group.

Correlations between fermentative power, on the one hand, and habitat, chromogenesis and liquefaction of gelatin, on the other, are indicated in table 5.

There is a marked tendency for the strains isolated from pathogenic conditions to ferment rather strongly, 76 (73 per cent) of all such strains attacking all four carbohydrates; while of the 54 strains from dust and air only 7 (13 per cent) attacked all four sugars and 30 (56 per cent) belonged to the heterogeneous feebly fermenting, group III.

Gelatin liquefaction was also slightly but distinctly more common among the active fermenters (60 per cent) while the members of group III were predominantly non-liquefiers (only 37 per cent showing liquefaction).

White and orange pigments on the other hand were fairly evenly divided among the various fermentative groups with a slightly greater preponderance of vigorous fermenters in the orange than in the white groups.

TABLE 5

General characteristics of 180 strains

	GROUP I. GLUCOSE, MALTOSE, SUCROSE, LACTOSE FERMENTED	GROUP II. GLUCOSE, MALTOSE AND SUCROSE FERMENTED	GROUP III. ALL OTHER STRAINS
Habitat			
Pathogenic strains	76	17	11
Strains from hands	7	7	8
Strains from air, dust and other external habitats	7	17	30
Chromogenesis			
White	43	28	29
Orange	47	13	20
Gelatin			
Liquefied	64	19	18
Not liquefied	26	22	31

REACTIONS IN MILK

In addition to the study of the sugar broths the reactions of the complete series in litmus milk have been determined with the following results:

Seventy-five strains acidified and clotted the milk and subsequently liquefied the clot.

Sixty strains acidified the milk, generally with clotting, but showed no subsequent liquefaction.

Seven strains showed no appreciable change in reaction.

Twenty-two strains turned the milk alkaline and liquefied the casein.

Sixteen strains turned the milk alkaline without liquefaction.

Dudgeon (1908) obtained somewhat lower results, 6 per cent of his orange and 48 per cent of his white strains clotting milk and 2 and 7 per cent respectively of the two groups peptonizing the clot.

Acid production in milk was, as might be expected, closely correlated with acid production in lactose broth as shown by table 6.

TABLE 6

Reactions in milk compared with reactions in lactose broth

LACTOSE BROTH	ACID	NEUTRAL	ALKALINE	TOTAL
(+) Acid	94	2	1	97
(±) Neutral	29	5	27	61
(−) Alkaline	12	—	10	22
Total	135	7	38	180

TABLE 7

Gelatin liquefaction compared with liquefaction of casein

LIQUEFACTION OF CASEIN	+	−	TOTAL
+	68	29	97
−	33	50	83
Total	101	79	180

TABLE 8

General grouping of staphylococci studied

FERMENTATIVE POWERS	ACTION IN GELATIN	NUMBER OF STRAINS IN EACH CLASS	
		Chromogenesis	
		White	Orange
Glucose maltose, sucrose and lactose all fermented	Liquefied	23	41
	Not liquefied	20	6
Glucose, maltose and sucrose positive lactose negative	Liquefied	10	9
	Not liquefied	18	4
Miscellaneous results, not falling in above groups	Liquefied	13	5
	Not liquefied	15	15

The liquefaction of casein was correlated to some extent, but not very closely, with the liquefaction of gelatin as shown in table 7.

Combining the results of tests for fermentation, gelatin liquefaction and chromogenesis we obtain the subdivision of our strains shown in table 8.

PRODUCTION OF INDOL, AMMONIA AND NITRITES

Indol production was studied in a medium containing 1 per cent peptone and 0.5 per cent K_2HPO_4 incubated at 20° for ten days and tested by the para-dimethyl-amido-benzaldehyde method. All tests were negative.

Ammonia production was determined in a medium containing 1 per cent peptone, 0.5 per cent NaCl, 0.05 per cent K_2HPO_4 and 0.01 per cent Na_2CO_3 incubated at 30° for seven days and tested with Nessler reagent. This test was suggested by Kligler (1913) as perhaps of special differential value, but in the present study all but 11 strains gave positive results.

The reduction of nitrates was first tested in a medium containing 1 per cent peptone, 0.5 per cent K_2HPO_4 and 1 per cent KNO_3. Incubation periods of seven days, fourteen days, and incubation temperatures of 30° and 37°, gave almost uniformly positive results. At 30°, 8 strains only out of 180 were consistently negative, while 19 more gave variable or doubtful readings.

This last result seemed somewhat surprising since the senior author in the Systematic Relationships of the Coccaceae reported only 21 per cent of the orange cocci studied and 13 per cent of the white strains as reducing nitrates. In 1908 when this earlier work was done the standard method of testing for nitrate reduction prescribed by the Committee on Standard Methods of Water Analysis of the American Public Health Association involved the use of a medium containing only 0.1 per cent peptone and 0.02 per cent nitrate. In order to see if this difference would explain the conflicting results we repeated our tests with the old medium but even here we found after seven days at 20° 132 strains clearly positive, 38 clearly negative and 10 doubtful or variable.

Gordon (1906) in his exhaustive study of the white staphylococci based one of his types on failure to reduce nitrates and the Winslows attributed this property to *Aurococcus aureus*, *Auro-*

coccus aurantiacus, and *Albococcus pyogenes*. In view of the results here reported we are inclined to suspect that these earlier results were perhaps due to the imperfections in technique which Conn and Breed (1919) have shown to have been so common in the past. In the small series of laboratory strains studied by Kligler only 4 out of 11 orange strains failed to reduce nitrates although 11 out of 12 results with white strains were negative.

CONCLUSIONS IN REGARD TO THE JUSTIFICATION OF A GENERIC DISTINCTION BETWEEN THE ORANGE AND WHITE STAPHYLOCOCCI

In view of the general results of this study of 180 strains of staphylococci we are forced to conclude that the generic distinction between the white and orange staphylococci previously suggested by the senior author is of doubtful validity. There is indeed a slight difference in liquefying power between the two chromogenic groups and of course, as is well known, a considerable difference in pathogenic power. In view, however, of the similarity between the orange and white pigment formers in all the other characteristics studied it seems on the whole most reasonable to consider them as belonging to a single generic group which, according to the citations of Buchanan (1915) should obviously bear the name *Staphylococcus*. This genus may be defined as follows:

Genus, *Staphylococcus* (Rosenbach). Parasitic cocci. Cells in groups and short chains. Gram positive. Produce on agar good growth of orange color or abundant growth of porcelain white color. Glucose, maltose and sucrose generally, and lactose frequently, fermented without production of gas.

CONCLUSIONS IN REGARD TO THE SPECIFIC TYPES TO BE INCLUDED IN THE GENUS STAPHYLOCOCCUS

Gordon (1906) classified his white staphylococci on the basis of nine tests which included liquefaction of gelatin, coagulation and peptonization of milk, reduction of nitrates and neutral red and fermentation of lactose, maltose, glycerol and mannitol.

His commonest type found on the skin coagulated milk but did not peptonize it, liquefied gelatin, reduced nitrates and neutral red, and fermented lactose, maltose and glycerol but not mannitol. A second type common on the scalp was the opposite of the first in every respect except that it also reduced nitrates. A third type fermented maltose and lactose but gave negative reactions to the other seven tests.

The characteristics of these three types of Gordon's (which are of importance from the fact that they were based on the study of 300 strains) are indicated in table 9.

TABLE 9

Characteristics of Gordon's three types of white staphylococci

	COMMONEST FORM ON SKIN ST. EPIDERMIDIS-ALBUS	COMMONEST FORM ON SCALP	SECOND FORM IN ABUNDANCE ON SKIN
Gelatin	+	−	−
Milk, coagulation	+	−	−
Milk, peptonization	−	+	−
Nitrate reduction	+	+	−
Neutral red, reduction	+	−	−
Lactose	+	−	+
Maltose	+	−	+
Glycerol	+	−	−
Mannitol	−	+	−

The Winslows used the liquefaction of gelatin and the reduction of nitrates for establishing their specific types in both the orange and white series, *Aur. aureus* being defined as liquefying and non-reducing, *Aur. aurantiacus* as non-liquefying and non-reducing, and *Aur. mollis* as reducing; *Alb. pyogenes* as liquefying and non-reducing, *Alb. epidermidis* as liquefying and reducing and *Alb. candidus* as non-liquefying.

If our practically universal positive results in regard to nitrate reduction are correct, the differentiation based on this characteristic in the earlier investigations must be considered as of doubtful value. There remain chromogenesis, liquefaction of gelatin and fermentation of lactose as the chief differential characters available for classification, with coagulation of milk closely cor-

related with lactose fermentation and peptonization of the clot, much less closely correlated with liquefaction of gelatin.

The use of the three primary characteristics listed above for specific differentiation would seem to be justified by the fact that all of them show a distinct bimodal curve, as shown by the Winslows for chromogenesis and gelatin liquefaction, and in the present study, for lactose fermentation.

TABLE 10

Reactions of twelve groups of Staphylococci

GROUP	PER CENT OF EACH GROUP POSITIVE													NAME
	Orange pigment	Glucose	Maltose	Sucrose	Lactose	Salicin	Gelatin	Gram	NO_3	Clotted milk	Liquefy milk	Pathological habitat	Number strains	
1	0	100	100	100	100	13	100	96	100	70	22	70	23	St. epidermidis (also perhaps St. ureae)
2	0	100	100	100	100	5	0	100	95	68	31	81	20	St. candidus (also St. tetragenus)
3	0	100	100	100	0	40	100	90	90	20	20	60	10	
4	0	100	100	100	0	0	0	100	52	16	26	21	18	St. candicans
5	0	63	8	63	31	0	100	92	70	23	23	48	13	
6	0	69	12	62	12	6	0	100	52	31	31	25	16	
7	100	100	100	100	100	0	100	95	100	36	61	90	41	St. aureus
8	100	100	100	100	100	0	0	100	100	83	16	100	6	St. aurantiacus
9	100	100	100	100	0	11	100	100	100	44	44	77	9	
10	100	100	100	100	0	0	0	100	75	25	50	0	4	
11	100	40	0	20	20	0	100	100	40	20	60	20	5	
12	100	6	13	6	6	0	0	100	94	20	26	0	15	

The use of these three characteristics would give us the eight distinct groups shown in the upper two thirds of table 8 and the four groups in the lower third of the table which are characterized by more restricted and highly variable fermentative power. The detailed reactions of these 12 groups are presented in table 10.

In establishing types among bacteria it is obviously absurd to give a specific name to every combination of characters which may occasionally be met with and we must give proper weight to the frequency with which a given type is found in nature.

From this standpoint it is evident that by far the commonest homogeneous type among the staphylococci is that which includes the orange, liquefying, actively fermenting strains. Forty-one of our 180 cultures were of this type and as shown by the detailed characteristics presented in group 7 of table 10 these 41 strains were remarkably uniform in all their properties except the clotting and liquefaction of casein in milk. Of the 203 strains of staphylococci studied by the Winslows 126 were of this type (if we ignore the distinction based on reduction of nitrates). It seems quite evident that this organism is the most abundant form among the staphylococci and the natural center about which all the other types are grouped. Assuming the distinction between *Aur. aureus* and *Aur. mollis* on the basis of nitrate reduction to be unwarranted, this central type of the staphylococcus group should certainly bear the name *Staphylococcus aureus;* and all the other types of the genus may be assumed to have been derived from this one by the loss of one or more of the characteristics of the primitive form.

Among the types which have retained the orange pigment production but which fail either to liquefy gelatin or to ferment lactose, none has occurred with sufficient frequency in the present series of strains to appear deserving of specific rank (see table 8 and groups 8 to 12 in table 10). Dudgeon's orange chromogens were also practically all alike in liquefying gelatin and fermenting lactose. On the other hand the Winslows found 49 strains in their series which produced orange pigment but failed to liquefy gelatin or ferment lactose identifying the type as *Aur. aurantiacus.* We may perhaps recognize this species (group 8, table 10) as *Staphylococcus aurantiacus* leaving the other two types, characterized respectively by gelatin liquefaction and failure to ferment lactose, and by lactose fermentation and failure to liquefy gelatin, (as well as the groups characterized by miscellaneous fermentative reactions) without specific names.

The series of white pigment formers appears from all the investigations to be much more variable in its reactions. Of the six groups represented in the third column of table 8 three are represented by 18 or more strains out of the 100 white strains

studied by us; and it is significant that these three types correspond to the three types of white staphylococci described by Gordon (compare table 9).

The most abundant form in each investigation was the type which fermented lactose and liquefied gelatin, called by Gordon *Staphylococcus epidermidis albus* and by the Winslows *Alb. epidermidis* and *Alb. pyogenes* (according as nitrates were or were not reduced). This type (group I, table 10) may best perhaps be known as *Staph. epidermidis* since it was Gordon's work which first made its characteristics clear. Our forms of this series agree with those of Gordon in generally coagulating milk and generally failing to peptonize the clot; though the agreement is by no means very close. Several cultures which came to us as *St. ureae* belong in this group. They acidify milk and produce ammonia.

The second type in abundance in our study, and the type found most commonly on the skin after *St. epidermidis* by Gordon, is the form which ferments lactose but fails to liquefy gelatin, identified by the Winslows as *Alb. candidus* and now to be called *St. candidus* (group 2 of table 10). Our strains, however, reduced nitrate and generally clotted milk which Gordon's type did not. Three strains sent in to the Museum collection as *M. tetragenus* all belong to this group. None of them reduce nitrates and results are variable in milk and in regard to ammonia production.

Finally the third form found commonly in the present study resembles Gordon's form characteristic of the scalp, which neither ferments lactose nor liquefies gelatin. This is the type for which Kligler suggested the name *Albococcus candicans*, and which should be called *St. candicans* (group 4 of table 10). Our strains however differed from Gordon's in failing to peptonize casein or ferment mannitol and in fermenting maltose.

Two of the strains in this group came to us originally as *M. neoformans*, both being alkaline in milk with variable results in regard to nitrate reduction.

The lactose-negative gelatin-positive type of white pigment producers appears in our study, as in that of Gordon, to be a

rarer one and this form, as well as the forms which exhibit miscellaneous fermentative reactions (see table 8) may best be left for the present without specific names.

The relations of the five principal types of staphylococci discussed above may be summarized as follows:

A. Orange Pigment
  a. Lactose fermented, gelatin liquefied
    St. aureus Rosenbach, Type, No. 4 of American Museum collection
  b. Lactose not fermented, gelatin not liquefied
    St. aurantiacus Schröter, Type, No. 348 of American Museum collection

B. White pigment
  a. Lactose fermented, gelatin liquefied
    St. epidermidis Gordon, Type, No. 25 of American Museum collection. *St. ureae* Cohn, No. 464 of the American Museum collection is apparently identical with *St. epidermidis*
  b. Lactose fermented, gelatin not liquefied
    St. candidus Cohn, Type, No. 49 of American Museum collection
    St. tetragenus Gaffky which may be differentiated from *St. candidus* by characteristic grouping of cells in fours and by viscid growth belongs in the same group, (No. 209 of American Museum collection)
  c. Lactose not fermented. Gelatin not liquefied
    St. candicans Flügge, Type, No. 526 of American Museum collection

In a group so variable as the staphylococci, it is clear that the conception of species has only a limited value, although it is convenient to have names associated with the types of most frequent occurrence. Fundamentally we are inclined to agree with Dudgeon in considering the whole group a reasonably homogeneous one; and it seems clear that the central type of the whole genus is the orange-pigment-forming, vigorously-fermenting, gelatin-liquefying, somewhat actively pathogenic *St. aureus*. As we depart from this type there is a progressive weakening of the various biochemical activities of this more vigorous form. The loss

of one characteristic of the *St. aureus* type tends in some degree to be associated with the loss of others. Thus the white chromogens are less actively pathogenic than the orange forms, less actively gelatinolytic, and slightly less vigorous in fermentative action. The forms which fail to liquefy gelatin also tend to be less active fermenters than the liquefiers (table 5).

Finally in considering the significance of these relationships, it must be pointed out that while the more typical and more vigorous orange-pigment producing gelatin-liquefying, lactose-fermenting staphylococci are the characteristic forms found associated with pathological conditions the types of weaker biochemical powers are the ones most frequently isolated from air and dust and other sources outside the human body. This correlation is brought out in Table 5 and is made clear by an inspection of the individual data presented in Table 10. It may plausibly be explained on the assumption that the loss of the bio-chemical powers characteristic of the typical *St. aureus* is promoted by the unfavorable conditions of life outside the human body.

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A SPECTROPHOTOMETRIC STUDY OF THE "SALT EFFECTS" OF PHOSPHATES UPON THE COLOR OF PHENOLSULFONPHTHALEIN SALTS AND SOME BIOLOGICAL APPLICATIONS[1]

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Contribution from the New York State College of Forestry and Syracuse University

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INTRODUCTION

This study was undertaken in connection with the investigation of the physiological, physical and chemical factors governing the growth of fungi and bacteria on culture media, trees, pulp wood, lumber, wood pulp and other cellulosic materials. In order to study the nature and habits of the fungi and bacteria causing the decay of wood and pulp and to devise and put into application methods of control, it is necessary to have accurate data on each organism concerned. Not only must the organisms be identified in pure cultures, but the maximum, optimum and minimum values of each essential physical, chemical and physiological factor must be established. For example, each organism living on an aqueous medium, and therefore in contact with hydrogen and hydroxylions (and other chemicals), will die if the concentration of the hydrogen ions (or other chemicals) is too large or too small, and will have its optimum growth when the hydrogen ion concentration is somewhere between the maximum and minimum values of tolerance, this statement, of course, presupposing that all other essential factors are constant

[1] This article is one that we are publishing in coöperation with Dr. Haven Metcalf, in charge of Forest Pathology, Bureau of Plant Industry, Department of Agriculture, on the quantitative studies of the various physical and chemical factors governing the growth of fungi and bacteria on culture media and cellulosic materials.

or optimum in value. We have already established some of these limiting values of ionic concentrations for several wood destroying fungi[2] and have begun to put the results into practical application in culturing the fungi, and in preventing the decay of chestnut trees[3] and of wood pulp,[4] for example.

The concentration of the hydrogen ions is a very important physiological and chemical factor governing the growth of wood destroying fungi. It is therefore essential to devise methods for measuring this factor accurately, especially as a very great effect on the growth is produced by small changes in hydrogen ion concentration in the regions of maximum and minimum values of tolerance. This is well illustrated in figure 1, which gives one of the many sets of experiments worked out by Meacham which will be reported later.

The hydrogen electrode[5] and sulfonphthalein indicators[6] were developed by Desha, Loomis, Myers, W. F. Clark, Slagle, White, Lubs, W. M. Clark, Guy, Birge, Hopfield and the writers for measuring hydrogen ion concentrations in solutions of weak and strong acids and bases, in the presence of salts, and in general under conditions such as we find in culture media. These methods are probably the best known for measuring the hydrogen ion concentration, but the presence of certain salts has an appreciable effect on the apparent values obtained by both methods. As the electrometric and colorimetric methods give different results, it is necessary to learn the magnitude of the errors in-

[2] See preliminary report: Meacham, Science, **48**, 499.

[3] Ibid. and several reports appearing later.

[4] See address by Acree before Buffalo Convention of Tech. Assn. Pulp and Paper Industry, published in *Paper*, June, 1919.

[5] Science, **30**, 624. Loomis and Acree: Am. Chem. J., **46**, 585, 621. J. Am. Chem. Soc., **38**, 2391. Desha and Acree: Am. Chem. J., **46**, 638. Myers and Acree: Ibid, **50**, 396. Clark, Myers and Acree: J. Phys. Chem., **20**, 243.

[6] Acree: Am. Chem. J., **37**, 72; **39**, 155, 528. Slagle and Acree: Ibid, **42**, 115, and unpublished data on phenolsulfonphthalein. White and Acree: Science, **42**, 1101; J. Am. Chem. Soc., **39**, 648; **40**, 1092; **41**, 1190. White and Davis: Jour. of Urology, **2**, 107. Lubs and Acree: J. Am. Chem. Soc., **38**, 2773, Lubs and Clark: J. Wash. Acad. Sci., **5**, 609; **6**, 483. J. Bact., **2**, 1, 109, 137. Birge and Acree: J. Am. Chem. Soc., **41**, 1031. Brightman, Hopfield, Meacham and Acree: Ibid, **40**, 1940.

herent in each, and to devise means of expressing the true hydrogen ion concentration, or activity, in terms of the results given by the two methods. We have therefore begun an investigation of these so-called "salt effects" by the use of a number of methods,[7] and as phosphates are cheap and efficient chemicals for regulating hydrogen ion concentrations, we are presenting here some data showing that increasing concentrations of such phosphates pro-

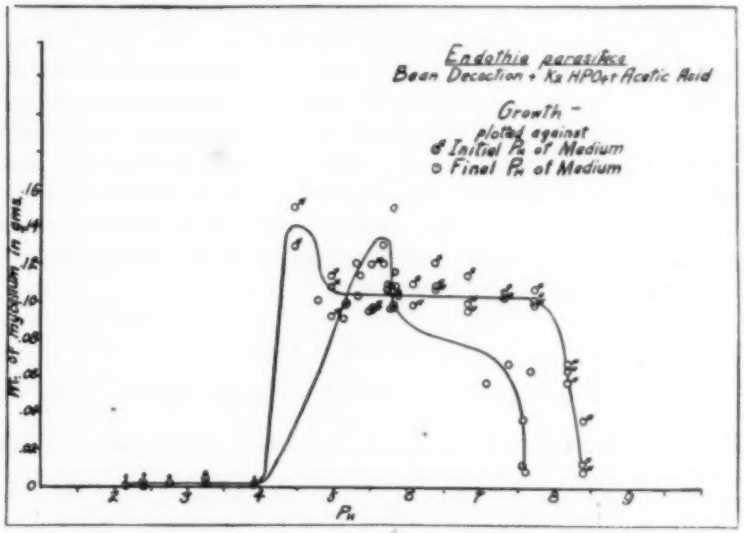

FIG. 1

duce increasing differences between the hydrogen ion concentrations indicated by the hydrogen electrode readings and by the colors of the indicators. These and other more extensive meas-

[7] Brunel and Acree: Am. Chem. J., **36**, 120. Acree: Ibid, **41**, 457. Loomis and Acree: Ibid, **46**, 5861, 632. Loomis, Meacham and Essex: J. Am. Chem. Soc., **38**, 2310; **39**, 1133. Acree and Slagle: Am. Chem. J., **42**, 130. White and Acree: J. Am. Chem. Soc., **40**, 1094; **41**, 1212. Lubs, Cloukey and Acree: Ibid, **38**, 2773, 2784. Mayer and Hantzsch: Ber. d. chem. Ges., **40**, 3479; **41**, 2568. Szyszkowski: Z. Physik. Chem., **58**, 420; **63**, 421; **73**, 269. Arrhenius: Ibid, **1**, 110; **11**, 823; **31**, 197. Harned: J. Am. Chem. Soc., **38**, 1916, and many other excellent papers. See also many papers in Amer. Chem. J. and J. Am. Chem. Soc., by Acree and coworkers on salt effects in reaction velocities.

urements now in progress show that reliable data on hydrogen ion concentrations will be secured only by using the isohydric principle, conductivity methods, the hydrogen electrode and the spectrophotometer with solutions of buffer salts and indicators having varying concentrations and increasing per cent of neutralization up to 100 per cent for both the indicator and buffer salts.

As such a study is very comprehensive and as other workers may be interested in knowing whether, and to what extent, phosphates show "salt effects" with phenolsulfonphthalein, we shall present here a provisional graphical method for showing the magnitude of the "salt effect" in a few cases and its influence on the apparent hydrogen ion concentration and ionization constant of this indicator. The application of these results to investigations of soils, wood extracts, biological fluids of all kinds, foods, industrial liquids and solutions of every description, will naturally occur to those interested.

EXPERIMENTAL WORK

When a tautomeric monobasic indicator is ionized[8] we have the following expression $K = \frac{H \times \alpha}{1-\alpha}$ where K is the apparent ionization constant of the indicator acting as an acid, α is the concentration of the colored anions, H is the hydrogen ion concentration and $(1-\alpha)$ is the concentration of the non-ionized molecules.

If we assume that the color of the indicator solution is a measure of the amount of the indicator which has been ionized, then it is possible to determine the value of $(1-\alpha)/\alpha$ colorimetrically. For example, if we use monosodium phenolsulfonphthalein as a monobasic indicator, we find that it shows an absorption band with a maximum for light of wave length $\gamma = 0.5557\ \mu$. In the future we shall refer to this band as the green band.

If light of intensity I_0 falls upon a cell containing a solution of the indicator, and the intensity of the transmitted light is I, then

[8] For fuller discussion of the quinone-phenolate theory applying to the sulfonphthaleins see references 6 and 7.

the ratio of the two intensities can be expressed by $\frac{I}{I_0} = 10^{-N}$. The term N we shall call the absorption index. If the absorption index for the middle of the band, when the indicator is partly ionized, is N_α, and the index when the indicator is completely ionized is N, and we assume the absorption to be proportional to the ionization, then $(1 - \alpha)/\alpha = (N - N_\alpha)/N_\alpha$ and if the hydrogen ion concentration (H) were known then $K = \frac{H \times N_\alpha}{N - N_\alpha}$. It is well known[9] that the presence of neutral salts affects the color of some indicators. It is then impossible to determine K directly if disturbing neutral salts are present. It is possible, however, to make solutions of approximately the same hydrogen ion concentration and yet vary the concentration of the salt. By determining the value of $(1 - \alpha)/\alpha$ due to the successive concentrations of the salt we can extrapolate and find the value that $(1 - \alpha)/\alpha$ would have were there no salt present, and, from that and the known hydrogen ion concentration, we can find the value of the ionization constant of the indicator and its variation with changing concentration of salt.

The method adopted was as follows: Three solutions were made up of sodium phosphate and alkali in such a manner that the amount of phosphate salt in each was the same (N/2). The concentration of the phenolsulfonphthalein was N/5000, but the values of the hydrogen ion concentration were different in each solution. These solutions were called A, B and C. To 100 cc. of these solutions 50 cc. of N/2500 phenolsulfonphthalein were added and the whole made up to 200 cc. with conductivity water. These solutions were denoted by A_2, B_2, and C_2, and are N/4 in concentration. To 100 cc. of these solutions 25 cc. of N/2500 phenolsulfonphthalein were added and the whole made up to 150 cc. with conductivity water. These were denoted by A_3, B_3, and C_3, and are N/6 in concentration.

The absorption index at the middle of the green band for each of these solutions was then measured by means of a Brace spec-

[9] For literature see reference 7.

trophotometer. The measurements are therefore more accurate than ordinary colorimetric data. The actual data and calculations involved are of course very extensive. We shall not give all these numerical values in this paper but state how the final figures given in the tables were obtained. The method was to measure the absorption for several wave lengths in the band. A smooth curve was drawn with wave lengths as abscissae and absorption indices as ordinates. The ordinate with the maximum value on this curve was taken as the absorption index for the middle of the green band. This index would change, of course, for different depths of cell and for various concentrations of the indicator. In order, therefore, that Birge's work might be utilized,[10] the value of the index was reduced, by Beer's law, to the value it would have were the cell 6 cm. deep and the concentration of the phenolsulfonphthalein N/1000. The values of the index (N_α) thus obtained are given in table 1, column 3.

Birge found in his work that, with different dilutions, the value of the absorption index for the green band when the indicator was wholly transformed showed some slight variation which will not appreciably modify the results in this paper. The value given for N/5000 concentration was 225. This was then taken provisionally for our value of N but will be measured for each concentration of the different indicators and of the phosphate and other buffers. Column 4 in the table gives the values of $(1 - \alpha)/\alpha$ as computed from the values $(N - N_\alpha/N_\alpha$.

The hydrogen ion concentration (H) of each solution was measured by the Loomis-Acree method[11] involving the N/10 KCl-HgCl electrode, 4.1NKCl as a connection link and the hydrogen electrode, and recorded in column 5 of the table. Column 6 gives the value of the ionization constant as computed from the values of $(1 - \alpha)/\alpha$ and (H). It will be seen that the value of K

[10] Unpublished work by Birge, Brightman, Meacham, Hopfield and Acree. See also Birge and Acree: J. Am. Chem. Soc., **41**, 1031, Brightman, Hopfield, Meacham and Acree, Ibid., **40**, 1940.

[11] Loomis and Acree: Am. Chem. J., **46**, 585, 621, Myers and Acree: Ibid, **50**, 396. Clark, Myers and Acree, J. Phys. Chem. **20**, 243. The errors involved in the Bjerrum method, and in the Walpole modification, which was used by Lubs and Clark, will be discussed in another article.

is far from constant and increases with increasing concentration of the salt, but averages about 2.65×10^{-8}.

In order to better appreciate this relation the points were plotted on coordinate paper, using values of H as abscissae and corresponding values of $(1 - \alpha)/\alpha$ as ordinates (fig. 2). Then

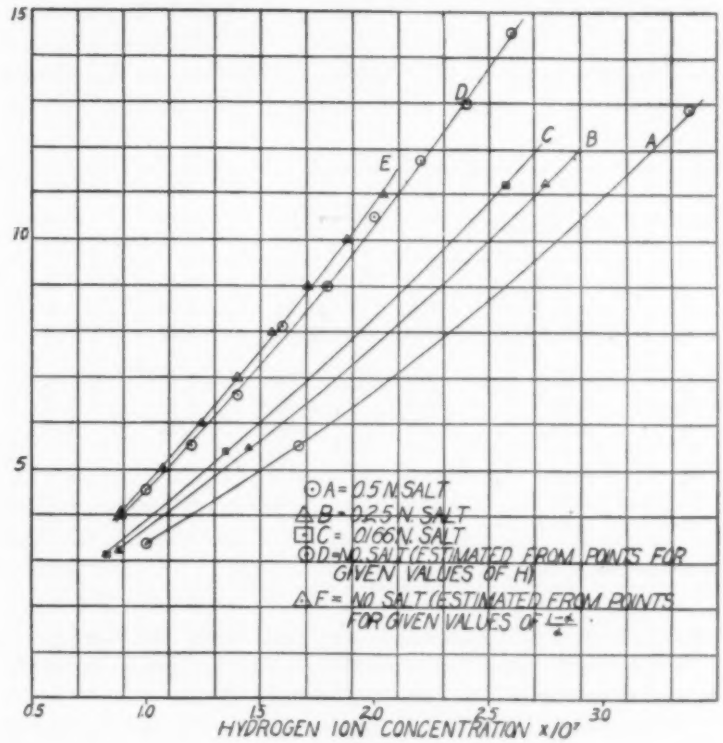

FIG. 2

the smooth curves A, B, and C, were drawn through the points which represented the same concentration of salt in the solution. These curves were then treated as follows: for values of $(1 - \alpha)/\alpha$ equal to 4, 5, 6, 7, 8, 9, 10 and 11 the change in hydrogen ion concentration for changes in concentration of salt from 0.5 N to 0.25 N and from 0.25 N to 0.166 N, were measured on the curves.

The average ratio of these two values was computed, and from this ratio we computed the value of the change in hydrogen ion concentration when the salt concentration changes from 0.166 N to 0. These changes were measured off on the curve sheet, the points being indicated by Δ, and a curve E was drawn through these points. The slope of this line can be called the ionization constant of the phenolsulfonphthalein. The value as determined from this curve is 1.93×10^{-8} (table 2). In the same way Curve D was obtained by taking given values of hydrogen ion concen-

TABLE 1

(1) SOLUTION	(2) SALT CONCENTRATION	(3) N_α	(4) $\frac{N-N_\alpha}{N_\alpha}$	(5) $H \times 10^7$	(6) $K \times 10^8$
A_1	0.5 N	16.2	12.88	3.39	2.63
A_2	0.5 N	18.4	11.23	2.75	2.44
A_3	0.5 N	18.4	11.23	2.57	2.28
B_1	0.25 N	34.2	5.54	1.67	3.02
B_2	0.25 N	34.8	5.46	1.46	2.67
B_3	0.25 N	35.2	5.39	1.35	2.50
C_1	0.16 N	51.5	3.37	1.00	2.96
C_2	0.16 N	53.3	3.22	0.88	2.73
C_3	0.16 N	54.0	3.16	0.83	2.63
Average value.....					2.65

tration between 10^{-7} and 2.6×10^{-7} and using the changes in $(1 - \alpha)/\alpha$ freed from "salt effects." The ionization constant obtained from curve D is 1.98×10^{-8} (table 3).

If this graphical method is approximately correct, curves D and E should coincide and the corresponding ionization constants should be identical. That such is found to be approximately true gives confidence in this method as a provisional one. The decrease in ionization constant is about that expected from our theory of these indicators and justifies the use of the simplified equations given above in place of the more complicated ones actually applying to the dibasic sulfonphthalein series. The decrease in ionization constant with decrease in sodium and other

applications of the isohydric principle and "salt effects," etc., will be studied in the future.

TABLE 2

(*Curve E*)

$(1-\alpha)/\alpha$	$H \times 10^7$	K
4	.89	2.21
5	1.08	2.16
6	1.24	2.06
7	1.41	1.98
8	1.56	1.95
9	1.61	1.79
10	1.69	1.69
11	1.74	1.58
Average value.....		1.93

TABLE 3

(*Curve D*)

$H \times 10^7$	$(1-\alpha)/\alpha$	K
1	4.6	2.17
1.2	5.5	2.16
1.4	6.62	2.12
1.6	8.15	1.96
1.8	9.00	2.00
2.0	10.5	1.90
2.2	11.75	1.87
2.4	13.00	1.84
2.6	14.55	1.79
Average value.....		1.98

CORRECTIONS FOR "SALT EFFECTS"

The bacteriologist and botanist, as well as the chemist, are interested in knowing the error in measurement of hydrogen ion concentration caused by these "salt effects." It will be observed that in the above curves the indicator is changed into the deeply colored salt to the extent of from 8 to 30 per cent, which covers the best range for accurate colorimetric work. As stated above, the entire range will be given later. The percentage change in hydrogen ion concentration necessary to produce the same color intensity in the different concentrations of the phosphate solutions is practically independent of the color intensity in this range. In other words, between about 8 and 30 per cent change of the indicator into the colored salt, the deviation in P_H with changing phosphate concentration, but constant color intensity is practically independent of the value $(N - N\alpha)/N\alpha$ or per cent of indicator present as colored salt.

Another way of stating this is to say that for a given hydrogen ion concentration more phosphate produces more color in the solution or a greater positive "salt effect," while more sodium produces less color. Now the magnitude of this "salt effect" is

not large and the "corrections" are small. If there is any change in this "correction," it fortunately becomes smaller with increasing color. These facts have been known *qualitatively* to Acree from preliminary work ever since this study of the sulfonphthalein series was suggested by him and undertaken by Slogle, White, Lubs, Clark, Guy, Birge, Hopfield and the present authors; it was one of the chief reasons, along with the intense color[12] of these indicators and the lack of "fading" in excess[13] of alkali, for choosing this as the most promising series of indicators to develop for colorimetric work. Bacteriologists not familiar with the history of the work are referred to the articles in the Journal of the American Chemical Society. Now that we have the present study completed, we can express *quantitatively* in the following table the "corrections" which workers must use to compare their P_H values for the same color in different phosphate concentrations. Any worker can calculate the "correction" to be applied to his own data in order to compare his results with those for another concentration of phosphate or even for cases where no phosphate is present. The table gives the corrections in P_H which must be *added* to the observed values in order to give the P_H in solutions free from phosphates and "salt effects" and having the same indicator transformation. The corrections are calculated from the above curves and the unpublished ones and can be considered accurate within the usual experimental errors. There may be some changes for the dilute solutions. As it requires careful work to determine hydrogen ion concentrations within 0.02–0.03 P_H it is clear that the "salt effects correction" for these phosphates below N/10 or N/25 concentrations is within the usual experimental error. As the writers have obtained excellent results in growing fungi on media buffered with M/25 and M/50 solutions of phosphates mixed with asparaginates, acetates and phthalates we recommend in general the use of the more dilute buffers in order to obviate these "salt" errors. Of course the concentration and "buffer range" required depends not only

[12] See the articles by White, Lubs, Birge and Acree, loc. cit., for the relation of the color intensity to the tautomeric equilibrium and ionization constants.

[13] See Brightman, Hopfield, Meacham and Acree, loc. cit.

on the limiting and optimum hydrogen ion concentration used by the organism, but also upon the concentration and ionization constant of the acid or base generated by the organism, and the proper selection can be made only after the preliminary study of the generated acid or base by (a) successive titrations with suitable indicators covering the P_H ranges involved or by (b) making a titration curve for the resulting medium with the aid of a hydrogen electrode. A fuller discussion of these points will be given in another article now completed.

TABLE 4

P_H *Correction to be added to colorimetric* P_H *for different concentrations of phosphate in order to obtain the electrometric* P_H

COLORED INDICATOR SALT	N/2 PHOSPHATE	N/4 PHOSPHATE	N/6 PHOSPHATE	N/10 PHOSPHATE	N/25 PHOSPHATE	N/50 PHOSPHATE	N/100 PHOSPHATE
per cent							
8.0	0.16	0.12	0.070	0.05	0.02	0.01	0.00
10.0	0.16	0.10	0.075	0.05	0.02	0.01	0.00
16.6	0.15	0.10	0.068	0.05	0.02	0.01	0.00
20.0	0.13	0.08	0.063	0.05	0.02	0.01	0.00
Rounded average	0.15	0.10	0.07	0.05	0.02	0.01	0.00

CONCLUSIONS

1. It is shown that phosphate solutions varying in concentration from N/2 downward and showing the same hydrogen ion concentration by the hydrogen electrode have marked influence on the color of phenolsulfonphthalein salts. This "salt effect" becomes small in phosphate solutions having low concentrations from 0.05 N down.

2. The apparent ionization constant of the phenolic group of the phenolsulfonphthaleins varies with the concentration of the phosphate present and averages about 2.65×10^{-8} when uncorrected for "salt effect."

3. When a graphical method is used for calculating the ionization constant freed from "salt effects" of phosphates, the value is lowered to about 1.95×10^{-8}.

4. In a more comprehensive study now in progress, the "salt effect" is under investigation for (a) all degrees of neutralization of several sulfonphthalein indicators and buffer salts from 0 to 100 per cent, and for (b) different concentrations of the indicators and buffer salts.

5. When measuring the limits of tolerance for hydrogen, or other ions, or molecules by organisms, care should be taken to state the concentration of chemicals and buffers used and possibly to free the published constants of these "salt effects."

A MODIFICATION OF LOEFFLER'S FLAGELLA STAIN

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Although the presence of flagella on motile bacteria was determined somewhat earlier, Robert Koch, in 1877, was the first to demonstrate their presence by the use of stains. He succeeded by using an aqueous solution of haematoxylin, and dilute chromic acid as a mordant. About 1889 Loeffler, by an improved method, succeeded in staining the flagella on a number of organisms whose flagella had previously not been demonstrated.

As it has been found that the arrangement of the flagella on bacteria is one of the characters which varies perhaps less than any other morphological characters, this arrangement furnishes an easy method for classification. Certain genera are separated on this one point alone as in the separation of the genus *Pseudomonas* from the genus *Bacillus* according to Migula's classification. Since the arrangement of flagella is thus used in establishing genera, it is necessary to determine definitely the character of the flagella on bacteria, and hence the need of reliable and simple flagella stains.

Loeffler, in his process, allowed the cover-glass preparation to dry, fixed it by passing through the flame, and then used the following solutions:

No. 1. Mordant

	parts
Solution of tannic acid (20 per cent aqueous)	10
Saturated aqueous solution (cold) of ferrous sulphate	5
Saturated alcoholic solution of basic fuchsin	1

No. 2. Stain

Carbol-fuchsin.

No. 3. Corrective solutions

A 1 per cent solution of caustic soda.

A solution of sulphuric acid of such strength that 1 cc. will neutraiize 1 cc. of the soda solution.

For certain organisms Loeffler claimed that no. 1 was sufficient, while in other cases varying amounts of the corrective solutions had to be added, according to the organism studied.

Since this method was devised by Loeffler, a number of modifications have come into use, one of the first being the abandonment of the corrective solutions, as their use was found to be unnecessary. Loewit (1896) modified the process by using a copper sulphate-tannic acid mordant instead of the ferrous sulphate-tannic acid. Another modification was made by Bunge (1894) who used ferric chloride in the mordant instead of ferrous sulphate. The use of ferric chloride in the mordant, in part, in my new modification is an application of Bunge's modification.

As a usual rule, flagella staining has been a more or less hit and miss process. In text-books on bacteriology it is in substance so acknowledged, as by Park and Williams (1914), who state that with the method advised "frequently the flagella appear well stained but often the process has to be repeated a number of times." Giltner (1916) states that "failure to make a good flagella stain with any method is no sign that the student is not a good workman, nor is success the sign of a good bacteriologist."

I have believed that at least a part of the difficulty of staining flagella has been due to difficulties in fixing the bacteria on the cover-glass. In most processes it is advised to heat the cover-glass preparation by passing it through the flame or by holding it over a flame, but it is sometimes added that care must be taken not to overheat. It is a difficult matter always to heat to the same extent by passing a cover-glass through a flame, and furthermore the flagella of certain bacteria might be overheated more readily than those of certain others.

As the fixing by heat has been one of the doubtful procedures, I have experimented with several different chemical solutions in order to accomplish the fixing without heat. Two solutions especially have been used, both of which have given good results, the preference being perhaps for the first, although both are effective. These solutions are made up as follows:

No. 1

Commercial formalin (40 per cent formaldehyde)	1
Distilled water	1

No. 2

Chromic acid (5 per cent. aqueous solution)	2
Acetic acid (10 per cent aqueous solution)	1

As soon as the cover-glass preparation, made as described later, has dried in the air, the cover-glass is flooded with either of these solutions, and after a minute and a half or two minutes it is washed off with water. Although I have had good results with both of these solutions, I have found that the mordant used by me is sufficient without previously using a special fixative so that it simplifies the process to dispense entirely with any fixing solutions.

It was my aim besides doing away with the heating of mordants and stains, so that the entire process is performed at room temperatures, to use only those solutions which will keep well, so as to avoid making them up freshly every time.

One of the first requisites for success in flagella staining is to have cover-glasses which are absolutely clean and free from every trace of oil or grease. I have found that they may be satisfactorily cleaned in the following manner: I drop them, one at a time, into a small stender dish containing a solution prepared as follows:

	parts
Water	1000
Potassium bichromate	60
Sulphuric acid (commercial)	60

After allowing the cover-glasses to stand in this cleaning solution for ten minutes, or longer, the solution is washed out by running clear tap water into the dish until every trace of color has disappeared. I then pour off the water and cover the cover-glasses with 95 per cent alcohol, using about 20 to 25 cc. of alcohol, and add about 15 drops of concentrated ammonium hydroxide. A few minutes later the cover-glasses are picked out, one at a time, with a pair of forceps, and dried carefully by wiping them with lens paper. It is best to use only one side of the paper,

as otherwise grease from the fingers may get on the cover-glasses which should never be touched with the fingers except around the edges. When wiped, the cover-glasses are placed in a well cleaned Petri dish and heated in an oven at a temperature of about 275 to 300°C. for one hour. In addition to these precautions I frequently flame the cover-glasses a little just before using.

In obtaining cultures to stain, it is usually necessary to have a culture eighteen to twenty hours old. However, in the case of certain organisms which multiply very rapidly, and which begin to form spores in a few hours, cultures not more than twelve hours old are sometimes desirable, as for example in the case of *Bacillus subtilis*. Certain other organisms which develop only at relatively low temperatures, and grow slowly, may give good results from much older cultures, even a week or more, as for example *Pseudomonas janthina*. For all of my work which has given favorable results, cultures on slanted agar-agar have been used.

The tube of slanted agar-agar is inoculated, and incubated at 37° if it will grow under those conditions; otherwise it is allowed to remain at room temperatures. When the culture is ten to twenty hours old or older, depending upon the species, some of the bacteria are carefully removed from the surface of the agar-agar, and placed in a test tube containing 2 or 3 cc. of sterilized tap water. Care is taken to remove none of the culture media while transferring the bacteria. It is best to have the tap water at the same temperature as the culture, and to accomplish this I usually place the tubes of sterile water in the incubator while the cultures are developing. After the transfer to the water, the tube is allowed to stand fifteen to twenty minutes, or longer, at room temperatures, or preferably in the incubator, in order to allow the bacteria to diffuse through the water, and to permit of some slight development.

Several small drops of this water are then placed on a clean cover-glass with a very small platinum loop, and without any attempt at spreading them, the drops are allowed to dry in the air. If the drops are small they will dry more rapidly. Good

results may be obtained from fairly large drops, but ordinarily they should not be over 2 or 3 mm. in diameter. As soon as the drops are dry, the preparation is ready for the mordant which is prepared as follows:

Solution A

	parts
Ferric chloride (1-20 aqueous solution)	1
Saturated aqueous solution tannic acid	3

This solution improves with age, and should be at least a week or two old. It should be kept made up in stock but filtered before using, although when using at frequent intervals, it need not be filtered except every few days.

Solution B

Anilin-oil	1
95 per cent. alcohol	4

About eight drops of solution A are placed on the cover-glass, this is followed immediately by one drop of solution B, and the combination is allowed to act at room temperatures for two minutes, then washed off with water, and the water drained off by touching the edge of the cover-glass to a piece of filter paper. The preparation should not be blotted as there is danger of scratching the film. Then the cover-glass is flooded with the stain which may be carbol-fuchsin, 1 per cent safranin in 50 per cent alcohol, anilin-oil-gentian-violet, or Loeffler's alkaline methylene blue. While I have had success with all of these stains, the stain which I prefer, and use most frequently is a special methylene blue prepared as follows:

	parts
Saturated alcoholic methylene blue	30
Potassium hydroxide (1:10,000)	100
Solution B of mordant	13

It is my practice to make up this stain by adding to 30 cc. of Loeffler's methylene blue, 3 cc. of mordant solution B. This stain is immediately ready for use and keeps well. The blue gives a color which is easier on the eye than the red of fuchsin or safranin.

The stain is allowed to act for two or three minutes at room temperatures, then the cover-glass is washed thoroughly with water, allowed to dry, and mounted in balsam.

The addition of the drop of mordant solution B to the eight drops of ferric tannate in solution A, gives a very fine precipitate which is largely responsible for the success of this method. Although I have been unable to prevent the precipitate from clinging to the glass to a greater or less extent, I have been able to demonstrate flagella with a remarkable regularity. In certain species it is usual to get flagella to show on practically every slide, and they are usually quite heavily stained. It is frequently possible to note the presence of flagella by the use of the 16 mm. objective of the microscope.

This process has given very good results in the hands of students who are doing only their third laboratory period's work on staining of any kind. Most of the class are able to get flagella in their first attempt, without any previous experience in flagella staining at all. I have found it possible, by shortening the time of allowing the mordant and stain to act, to demonstrate flagella, mounted in balsam, in a little less than five minutes from the time the bacteria were placed on the cover-glass.

I have modified the process slightly to see if it were possible to color the flagella differently from the cell wall. After allowing the action of the chrom-acetic fixing solution, and of the mordant, the preparation was stained with either Loeffler's methylene blue, or the special methylene blue for four or five minutes, then 1 per cent safranin in 50 per cent alcohol was applied for from two to four minutes, and the cover-glass preparation was washed, dried, cleared with cedar oil, and mounted in balsam. This modification of the process was tried mainly with *Bacillus vulgaris*, and gave fairly good results at times. The flagella appear red, the cell wall bluish, and the color of the protoplast is about the same color as that of the flagella. This gives further support to the contention that the flagella are not appendages of the cell wall, as they were formerly supposed to be, but rather continuations of the protoplast.

SUMMARY

This modification of Loeffler's method differs from previous modifications chiefly in the use of a solution of anilin-oil in alcohol (1:4) in connection with the ferric-chloride tannic acid

mordant which was Bunge's modification. By using one drop of this solution with about eight drops of the ferric tannate solution, applied together on the cover-glass, all necessity of heating the mordant or the stain is obviated.

The stain recommended is a special methylene blue made by adding an alcoholic solution of anilin-oil (1:4) to Loeffler's methylene blue in the ratio of 1:10.

The chief advantages of the process are (1) its simplicity, (2) the use of solutions that keep well, (3) the use of all solutions at room temperatures, and (4) the high percentage of successful attempts, even in the hands of inexperienced students.

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BOUILLON CUBES AS A SUBSTITUTE FOR BEEF EXTRACT OR MEAT IN NUTRIENT MEDIA

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During the war the high price of meat and the frequent scarcity of meat extract seriously hampered the preparation of ordinary nutrient media in large quantities, and ordinary bouillon cubes were therefore tested out as a possible substitute. As these cubes contain, besides beef extract, certain vegetable extracts, their use in media for some organisms might be of doubtful value, while for others the growth would be more or less favored. It was to determine this point that the following experiments were carried out. Bovim and Steero bouillon cubes were used in all the tests. Comparisons were made of cultures grown in similar broths made from each kind of cube.

Two lots of 500 cc. each of broth were prepared using Bovim and Steero cubes respectively in the proportions of one cube to 250, 500, 750, 1000 and 1500 cc. of water.

The cubes were dissolved in boiling tap water, the peptone added and dissolved; then the liquid was cooled to coagulate the fats present, and filtered cold, after which it was tubed and sterilized at 15 pounds in the autoclave.

Subsequent to sterilization two tubes of each dilution both with and without peptone were inoculated with the following organisms, *B. typhosus*, *B. prodigiosus*, *B. subtilis*, *B. coli* and *B. botulinus*. Results proved entirely satisfactory.

Since these experiment were concluded (nearly a year) the use of bouillon cubes as a substitute for meat extract in ordinary media has been general and has given entire satisfaction to all our laboratory staff. As a substitute for meat it is as satis-

factory if not more so, than the ordinary meat extracts. Our laboratory stock cultures, pathogens and all, with but few exceptions, are at present growing very well indeed on this bouillon cube medium. One or one and a half cubes (about 3 or 5 grams) are used per liter at a much decreased cost, with an increased ease in preparation, and the titer of the medium does not have to be changed unless so desired.

From our experiments and practical experience, it is quite evident that the use of ordinary bouillon cubes can be recommended in general laboratory work where ordinary beef extract or meat media has been formerly used. Their use is also suggested in the preparation of various special media.